PURIFICATION AND CHARACTERIZATION OF A LECTIN FROM *FUSARIUM* SP. LR11 HAVING COMPLEX SUGAR SPECIFICITY

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BY

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DEDICATED TO

MY GRAND FATHER

CONTENTS

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Feroz Khan

CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Purification and characterization of a lectin from *Fusarium* sp. LR11 having complex sugar specificity"** submitted by Mr. Feroz Khan was carried out under my supervision. Such material as has been obtained form other sources has been duly acknowledged in the thesis.

Dr. M. I. Khan
Research Guide

DECLARATION OF THE CANDIDATE

I declare that the thesis entitled **"Purification and characterization of a lectin from *Fusarium* sp. LR11 having complex sugar specificity"** Submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me during the period from **3rd Sep, 2001 to 9th March, 2006** under the guidance of **Dr. M. I. Khan** and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institute of Higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Signature of the Candidate Date . . . . . .

Feroz Khan

ABSTRACT

Lectins are carbohydrate binding proteins found in virtually all groups of living organisms. They find applications in (1) isolation of glycoproteins, (2) structural studies of carbohydrates, (3) identification of cell surface sugars and (4) detection of cell surface changes during differential, development and malignancy. The biological role of lectins is a consequence of their binding to the cell surface carbohydrates.

Fusarium is a well-known plant pathogen, and found to be involved in substantial loss of several economically important crops such as tomato, chickpea and soybean. High specificity of fungal lectins for complex glycans makes them useful in the biochemical study of membrane and cell wall glycoconjugates. Investigation was carried out to purify and characterize the *Fusarium* sp. lectin, and to determine its structure-function relationship. The thesis is divided into six Chapters.

Chapter 1 : General Introduction

This part comprises a literature survey of lectins with reference to their purification, properties and applications.

Chapter 2 : Purification and characterization of *Fusarium* lectin

A lectin from the mycelial extract of an endophytic strain of *Fusarium* sp. was purified to homogeneity by successive and repeated chromatography on Phenyl-Sepharose followed by gel filtration with an overall yield of 26%. The molecular mass of the lectin determined by gel filtration is 26 kDa and it is made up of two identical subunits of 13 kDa. It is a basic protein with a pI of 8.7. The purified lectin is a glycoprotein and contains 3.9% carbohydrate. It exhibited high pH stability and temperature stability. The partial N-terminal sequence did not show similarity with any known lectin. Purified lectin agglutinated neuraminidase/ pronase treated human erythrocytes (A, B and O), with very low titre values. Its hemagglutinating activity was inhibited by

glycoproteins containing N-linked or O-linked glycans. Chemical modification studies suggested the involvement of tyrosine in hemagglutinating activity.

Chapter 3 : Carbohydrate binding and solute quenching studies of the *Fusarium* lectin by fluorescence spectroscopy

The intrinsic fluorescence intensity of *Fusarium* lectin was quenched upon binding to disaccharides and glycans, without any change in the emission maximum (348 nm). The quenching observed with asialo-triantennary was 5%. The lectin interacted very poorly with monosaccharides but well with disaccharides indicating an extended binding site. Thermodynamic studies revealed that the binding of the lectin with all the saccharides is enthalpically driven and exothermic in nature. The asialo-triantennary glycan showed 5 fold higher affinity than asialo-biantennary glycan without any significant increase in binding enthalpy, pointing towards the importance of multivalency in the lectin-ligand interactions.

Solute quenching studies of the lectin using acrylamide, succinimide, potassium iodide and cesium chloride were carried out in the absence and presence of asialo-triantennary glycan and at different pH. This studies showed that the single tryptophan residue of the lectin (per monomer) is relatively exposed, and could be in the vicinity of positively charged amino acid residues.

Chapter 4 : Glycan binding to the *Fusarium* lectin: Surface plasmon resonance studies

The thermodynamics and kinetics of binding of glycans and glycoproteins to *Fusarium* sp. lectin was studied using surface plasmon resonance. The lectin showed very high affinity for asialo-bovine submaxillary mucin (K_a=1.61 × 10^{10} M^{-1} at 25 °C). The affinity for various glycoproteins were in the order of asialo-BSM >> BSM ≅ asialofetuin > fetuin ≅ asialofibrinogen > fibrinogen. Compared to glycans, their corresponding glycoproteins showed several fold higher affinity with significant contribution from enthalpy and positive

entropy, suggesting the involvement of non-polar protein-protein interaction. The higher affinity of the glycoproteins was due to their faster association rates and the activation energy in the association process was much lower for the glycoproteins than glycans, resulting in their faster associations. These observations elaborate a role of protein matrix in lectin-glycoconjugate interaction.

Chapter 5 : Denaturation and unfolding of *Fusarium* lectin

The conformational stability of *Fusarium* lectin was determined with chemical, thermal and pH denaturants. Equilibrium unfolding with guanidine thiocynate showed that the values of $D_{1/2}$ (0.49 M) and ΔG^{H_2O} (5.24 kJ/mol) were lowest at pH 12. The maximum conformation stability of *Fusarium* lectin was observed at pH 8.0 near its pI. At pH 12, the ΔG^{H_2O} and $D_{1/2}$ values of urea induced unfolding was higher than that of guanidine thiocynate. Curves of fraction unfolded (f_U) obtained with fluorescence and CD measurements overlapped at all the pH studied. Moreover, the denaturation of the lectin was irreversible at higher denaturant concentration. At acid pH, *Fusarium* lectin showed a flexible tertiary structure with pronounced secondary structure with retention of its hemagglutinating activity. Thermal denaturation did not result in aggregation or precipitation of the protein even at high temperatures. ANS binding studies revealed no exposure of hydrophobic patches.

Chapter 6 : General Discussion and Conclusion

This part compares the properties of *Fusarium* lectin with other lectins with respect to their biophysical properties.

List of abbreviations used

BSM	: Bovine submaxillary mucin
DEP	: Diethylpyrocarbonate
DTNB	: Dithiobisnitrobenzoic acid
Fuc	: L-Fucose
Gal	: D-Galactose
Galβ1→ 3GlcNAc	: 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-D-glucopyranose;
Galβ1→3GalNAc	: 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-D-galactopyranose
Galβ1→4GlcNAc	: 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-D-glucopyranose
GalNAc	: N-acetyl-D-galactosamine
Gdn-HCl	: Guanidine hydrochloride
Gdn-HSCN	: Guanidine thiocynate
Glc	: D-Glucose
GlcNAc	: N-acetyl-D-glucosamine
K_1	: Association rate constant
k_{-1}	: Dissociation rate constant
K_a	: Affinity constant
K_q	: Modified Stern Volmer quenching constant
K_{sv}	: Stern Volmer quenching constant
MALDI-ToF	: Matrix-assisted laser desorption ionization time-of-flight
Man	: D-Mannose
Me	: Methyl
NAI	: N-acetylimidazole
NBS	: N-bromosuccinimide
PAGE	: Polyacrylamide gel electrophoresis
PMSF	: Phenylmethyl-sulphonilfIuoride

SPR	: Surface plasmon resonance
TBS	: Tris buffer saline
WRK	: Woodward's reagent K

CHAPTER : 1

GENERAL INTRODUCTION

Lectins, a well-known class of multivalent carbohydrate binding proteins of non-immune origin which recognize diverse sugar structures with a high degree of stereospecificity in a non-catalytic manner, are wide spread in distribution (1). They have been implicated in cellular signaling, malignancy, host pathogen interactions, scavenging of glycoproteins from the circulatory system, cell-cell interactions in the immune system, differentiation and immune responses and protein targeting to cellular compartments (1-3). Goldstein (4) defined lectins as di- or multivalent carbohydrate binding proteins or glycoproteins of nonimmune origin which agglutinate cells and/or precipitate glycoconjugates. However, with the discovery of lectins with toxic or harmone like activities, this definition was found to be inadequate. To overcome these shortcomings, Kocourek and Horejsi (5) proposed a modified version of the definition as "Lectins are proteins of nonimmunoglobulin nature capable of specific recognition and reversible binding to carbohydrate moieties of complex carbohydrate without altering the covalent structure of any of the recognized glycosyl ligands". However, in the light of data, obtained mainly from molecular cloning of lectin and lectin related proteins, this broad definition was still inadequate. Hence, to overcome these shortcomings lectins were classified into three groups based on their overall structure and properties.

Merolectins : These are small proteins, incapable of precipitating glycoconjugates or agglutinating cells because of their monovalent nature. Monomeric mannose binding proteins from orchid (6,7) and class I chitinases that possess a chitin binding domain and a catalytic domain separated by hinge region (8) come under this family.

Hololectins : They are exclusively made up of carbohydrate-binding domains. However, unlike merolectins, they contain two or more carbohydrate-binding domains which are identical or very homologous and bind either the same or structurally similar sugars. Since hololectins have multiple binding sites they are fully capable of agglutinating cells

and precipitating glycoconjugates. Most plant lectins belong to this subgroup (9-11).

Chimerolectins : These are fusion proteins composed of a carbohydrate-binding domain tandemly arrayed with an unrelated domain with well-defined catalytic activity which functions independently of the former (5). Depending upon the number of binding sites, chimerolectins behave as merolectins or hololectins. For example, the type 2 Ribosome inactivating proteins (RIPs) ricin and abrin consist of a toxic A chain (which has the N glycosidase activity characteristic of all RIP's) and a carbohydrate binding B chain (12) with two carbohydrate binding sites to agglutinate cells function as hololectins whereas class I chitinases with a single chitin binding domain that do not agglutinate cell behave as merolectin (13).

Historical perspective

The lectin was first discovered by Stillmark in 1888 as an agent that can agglutinate animal erythrocytes from extract of castor bean (14). Boyd and Shapleigh in 1949 coined the term lectin (Latin, 'legere', to select or choose) based on their observation that some plant seed extracts could distinguish among human blood-groups (15-17). Hellin in 1891 discovered abrin (18), the toxic protein from jequirity beans. During the 1890's, Ehrlich worked with ricin and abrin, and discovered immunospecificity and reversibility of the antigen-antibody reaction as some of the fundamental principles of immunology. Landsteiner and Raubitschek in 1908 (19) found some lectins showing specificity for the red blood cells of certain species, while in 1945 Boyd and Reguera (15) established blood group specificity of the lectin. One of the first lectins to be crystallized was con-A from jackbean by Sumner (20). Sugar specificity and inhibition of hemagglutination of the lectin was discovered by Watkins and Morgan in 1952 (21). Researches on lectin gained momentum when Nowell's laboratory at the University of Pennsylvania, Philadelphia reported the mitogenicity of lectins, wherein the lectins

stimulated mitosis in the dead-end lymphocyte cells (22). During the same period, Joseph demonstrated that WGA and Con A lectin preferentially agglutinated the malignant cells.

Lectins are wide spread in distribution and have been isolated from microorganisms, plants and animals (Table 1.1). Although most of the well characterized lectins are from plants, fungal lectins are receiving increased attention due to their importance from basic as well as applied aspects. This compilation gives a comprehensive account of fungal lectins with respect to their occurrence, purification, physicochemical properties, biological roles and applications.

Table 1.1: Occurrence of lectins in different organisms

Lectin	Source	Reference
Virus		
Influenza viral lectin	Influenza virus	(23)
Myxo and paramyxo lectin	Myxo and paramyxo virus	(23)
Bacteria		
PA-IL	*Pseudomonas aeruginosa*	(24)
RSL and	*Ralstonia solanacearum*	(25)
hemagglutinin B (HagB)	*Porphyromonas gingivalis*	(26)
HBHA	*Mycobacterium tuberculosis*	(27)
FHA	*Bordetella pertussis*	(28)
Fungi		
PHA and PHB	*Agaricus bisporus*	(29)
BSL	*Boletus satanas*	(30)
CGL1 and CGL2	*Coprinopsis cinerea*	(31)
Ganoderma lectin	*Ganoderma capense*	(32)
Grifola lectin	*Grifola frondosa*	(33)
Hericium lectin	*Hericium erinaceum*	(34)
TML-1 and TML-2.	*Tricholoma mongolicum*	(35)

Lectin	Source	Reference
Plant		
TDSL	*Trichosanthes dioica*	(36)
Artocarpus lectin	*Artocarpus hirsuta*	(37)
Phaseolus vulgaris lectin	*Phaseolus vulgaris*	(38)
--	*Musa basjoo*	(39)
--	*Vicia sativa*	(40)
EspecL	*Erythrina speciosa*	(41)
Animal		
Anthocidaris lectin	*Anthocidaris crassipina*	(42,43)
Electricus lectin	*Electricus electricus*	(44)
--	*Craniella australiensis*	(45)
Anguilla lectin	*Anguilla rostrata*	(46)

FUNGAL LECTINS :

The first fungal lectin 'phallin' was reported by Kobert in 1891 from *Amanita phalloides*, which was a hemolytic agent (47). Later a fungal hemagglutinin was discovered in the fly agaric (48). *Aleuria aurantia* was the first fungal lectin, for which the crystal structure was solved by Wimmerova *et al.* in 2003 (49).

Occurrence and localization

The occurrence of lectins in fungi is wider than in higher plants (50). Fungal lectins have been isolated from mycelium (51), conidia (52), sporomes (53), basidiomes (54) and fruiting bodies (55) whereas, the lectin from the pathogenic fungus *Macrophomina phaseolina* is extracellular in nature (56).

Detection and assay

Lectins were earlier discovered as hemagglutinating agents; hence, the technique is universally used to detect the lectin in the past and even today.

The hemagglutination is carried out using animal as well as human erythrocytes (57-60). Sometimes erythrocytes are treated with enzymes like pronase, trypsin, papain and neuraminidase or any other proteolytic enzyme (60-63). Other types of cell lymphocytes, sperm, and yeast have also been used for lectin detection (59,64). Lectins also form cross-links between polysaccharide (65-68) or glycoproteins (69-73) in solution and induce their precipitation.

Purification

Since the majority of fungal lectins are intracellular, most of the purification procedures, involves steps like lysis of cells, isolation of different parts, concentration of the crude extract by either salt precipitation, ultrafiltration or lyophilization followed by conventional purification methods, such as ion-exchange chromatography and gel filtration.

Ion-exchangers like DEAE-, CM-cellulose and QAE-Toyopearl have been used for the purification of fungal lectins (74-76). Gel filtration has been used as one of the purification steps for lectins from *Rhizopus stolonifer* (77) and *Aspergillus fumigatus* (52). In addition, hydrophobic matrix like Phenyl-Sepharose has been employed for the purification of lectin from *A. fumigatus* (52) and *Beauveria Bassiana* (76).

Affinity chromatography has been extensively used for the purification of fungal lectins. The ability of lectins to agglutinate erythrocytes has been utilized for their purification on erythrocytes and erythrocyte stromas embedded in polyacrylamide (78). The other affinity absorbents include fetuin-Sepharose (56), BSM-Toyopearl (79) and gal-Sepharose (51). In some cases, Sephadex and Sepharose or acid treated Sepharose have been used to purify glucopyranosyl/mannopyranosyl and galactopyranoside binding lectins respectively, where they serve as affinity matrix and not as molecular sieves. Sepharose was used for the isolation of a lectin from *Ischnoderma resinosum*, specific for methyl β-galactoside (80).

Modern purification techniques like HPLC and FPLC have been successfully used for purification of lectins from *Hygrophorus hypothejus* (81), *Ganoderma capense* (32) and *Peziza sylvestris* (82).

Molecular mass and subunit structure

The molecular mass of fungal lectin range from 15-90 kDa, but majority of them are between 23-36 kDa (Table 1.2). Most of them are dimeric proteins and the subunits are held together by non-covalent interactions. Exceptionally, subunits of *Phallus impudicus* (83) and *Lactarius lignyotus* (84) are linked together by disulphide linkages.

Table 1.2. Physical properties of fungal lectins

Source	Molecular mass (kDa)	Subunit type	pI	Carbohydrate content (%)	References
Agaricus blazei	70	α_4		11	(85)
Arthrobotrys oligospora	36	α_2	6.5		(86)
Auricularia polytricha	23	α	10.6	3.5	(87)
Beauveria bassiana	15	α	7.1	12.6	(76)
Chlorophyllum molybdites	32	α_2	3.75	12	(88)
Clitocybe nebularis	30	α_2	4.3	10	(89)
Hygrophorus hypothejus	68	α_4	5.0	0	(81)
Ischnoderma resinosum	32	α_2	5.5	4	(80)
Laccaria amethystea	16	α	9.5	0	(78)
Lactarius deliciosus	37	$\alpha\beta$	6.7	0	(90)
Lactarius lignyotus	100	α_4	-	4	(91)
Macrophomina phaseolina	34	α	-	16.4	(56)
Peziza sylvestris	20	α	-	-	(82)
Pleurotus ostreatus	81	$\alpha\beta$	7.6	0	(92,93)
Pleurotus ostreatus	72	$\alpha\beta$	-	-	(93)

Source	Mole-cular mass (kDa)	Sub-unit type	pI	Carbohy-drate content (%)	References
Rhizoctonia solani	31	α_2	>9	0	(94,95)
Rhizopus stolonifer	28	α_6	--	--	(77)
Xerocomus spadiceus	32	α_2	-	-	(96)

Isoelectric point

The isoelectric points of the majority of fungal lectins are in the range of 5-8 (Table 1.2). Lectins from *Clitocybe nebularis* (89) and *Chlorophyllum molybdites* (88) are highly acidic proteins with a pI of 4.3, and 3.75, respectively whereas, the lectins from *Laccaria amethystea* (78) and *Auricularia polytricha* (87) are highly basic proteins with pI values of 9.5 and 10.6 respectively.

Carbohydrate content

The carbohydrate content varies from lectin to lectin and in some cases it could be high as 30% in *Rigidoporus lignosus* (97) while in some lectin it is totally absent as in *Laccaria amethystea* (78) (Table 1.2).

Metal ion requirement

Fungal lectins in general do not require metal ions for their activity. The lectin of *Xerocomus chrysenteron* require Mn^{2+} for its activity (84). In case of *Xerocomus spadiceus* lectin Zn^{2+} and Al^{3+} stimulated the activity (96) while Fe^{3+} stimulated the activity of *Polyporus adusta* lectin (55). On the contrary, Fe^{3+}, Al^{3+}, and Zn^{2+} inhibited activity of *Armillaria luteo-virens* lectin (98).

Specificity

Carbohydrate specificity

Fungal lectins exhibit a broad specificity varying from simple sugars to glycoproteins. *Peziza sylvestris* lectin is specific for arabinose while *Hericium erinaceus* lectin is specific for sialic acid (Table 1.2). Gallagher

classified fungal lectins into two groups according to their carbohydrate specificity (99).

a) **Exolectins:** Which bind to an appropriate external, nonreducing sugars in complex saccharides and their agglutination can be inhibited by low concentrations of the respective free sugar or their methyl glycosides. The first reported exolectins were PAL-I and PAL-II from *Phaeolepiota*. Other exolectins reported include those from *Agrocybe aegerita* (100), *Grifola frondosa* (101), *Clitocybe nebularis* (102), *Laccaria amethystina* (78), *Psathyrella velutina* (103).

b) **Endolectins:** Which recognize complex oligosaccharides and their agglutination can be inhibited only by specific sugar sequences. All *Agaricus* lectins can be defined as endolectins (29,104).

Table 1.2: Carbohydrate specificity of fungal lectins

Lectin	Specificity	Reference
Agaricus bisporus	Galβ1→3GalNAc, and glycoproteins	(29,104)
Agaricus blazei	Glycoproteins	(85)
Agrocybe aegerita	Glycoproteins	(100)
Aleuria aurantia	L-Fucose and glycoproteins	(105,106)
Aspergillus fumigatus	Glycoproteins	(52)
Auricularia polytricha	D-Galactose and lactose	(87)
Beauveria bassiana	Glycoproteins	(76)
Boletus edulis	Lactose	(107)
Boletus satanas	D-Galactose	(30)

Lectin	Specificity	Reference
Boletus subtomentosus	Lactose	(107)
Clitocybe nebularis	D-Galactose	(89)
Coprinopsis cinerea	Galactose	(108)
Coprinus atramentarius	Lactose	(107)
Flammulina velutipes	Glycoproteins	(109)
Fomes formentarius	D-Galactose	(89,110)
Grifola frondosa	Glycoproteins	(101)
Hericium erinaceus	Sialic acid and glycoproteins	(34)
Hericium erinaceus	Glycoproteins	(34)
Hygrophorus hypothejus	Lactose and glycoproteins	(111)
Hygrophorus hypothejus	Glycoproteins	(112)
Kuehneromyces mutabilis	Glycoproteins	(113)
Laccaria amethystea	L-Fucose, Lactose	(78)
Laccaria laccata	L-Fucose and glycoproteins	(112)
Lacterius deterrimus	Glycoproteins	(91)
Lactrius deliciosus	Galβ1→3GalNAc	(114)
Laetiporus sulphureus	D-Galβ→4D-GlcNAc	(115)
Lentinule edodes	D-Glucosamine	(116)
Macrophomina phaseolina	N-Acetylneuraminyl N-acetyllactosamine	(56)
Marasmius oreades	D-Galactose	(117)
Panus conchatus	D-Galactose	(118)
Peziza sylvestris	Arabinose	(82)
Pholiota squarrosa	L-Fucose	(112)
Pleurotus ostreatus	Lactose	(119,120)
Pleurotus spodoleucus	Lactose	(119,120)
Psathyrella lacrymabunda	N-Acetyl-D-glucosamine	(103)
Sclerotinia sclerotiorum	Lactose, Lactulose	(51)

Lectin	Specificity	Reference
Sclerotium rolfsii	Glycoproteins	(121)
Xerocomus chrysenteron	Glycoproteins	(84)

Cellular specificity

Lectins show specificity for certain cell types, *e.g.* erythrocytes, belonging to specific human or animal phenotypes and certain microbial cells. This property of the lectins has been used in the taxonomy of animal and single-cell plants.

Human erythrocytes : Some of the fungal lectins showed discrimination between erythrocytes of different blood groups. Treating erythrocytes with proteolytic enzymes can enhance their activity or modify specificity. The anti-H activity of *Laccaria proxima* lectin can be enhanced in this way, without modifying its specificity, by prior treatment of the cells with papain (53). Similar observation were made in *Clathrus cancellatus,* where enhancement in its anti-O effect was observed by papain treatment (122). In some cases higher specific activity was observed with trypsinized erythrocytes (123).

Sometimes, presence of macromolecules can facilitate agglutination and raise lectin activity, with or without alteration in specificity. Dextran and bovine albumin together with ficoll have been the most widely used (53,122).

Animal erythrocytes : It has been observed that certain fungal lectins can discriminate both between human and animal erythrocytes, as well as among different animal erythrocytes. The lectin isolated from *Mycena galericulata* agglutinates pig erythrocytes exclusively (123). Whereas an extract from *Irpex sinuosus* agglutinates only trypsinized horse and rabbit erythrocytes (124). Several other erythrocytes from mammals, birds, reptiles and amphibians have also been used for hemagglutination (34,75,107,110,124-129) .

Other types of cell : It has been reported that lectins isolated from few fungi do not find complementary structures for their binding sites on any type of erythrocytes tested but bind to other cells. This was observed for one agglutinin of *Russula nigricans* that is active on the yeast *Candida albicans* (111,130). Similarly, the presence of a non-erythroagglutinant lectin in *Clitocybe geotropa* has been demonstrated in a study of the glycocalix of the protozoan *Crithidia oncopelti* (131).

Effect of fungal lectin on different cells

Lectins are found to show other cell activities besides agglutination *e.g.* the binding of a lectin on a membrane receptor can trigger some specific cell reaction, or have a cytotoxic effect.

Effect on lymphocytes : Nowell in 1960 observed that some phyto-hemagglutinin, from bean and other plants including legumes, in addition to agglutinating activities towards lymphocytes, can also induce their conversion into lymphoblast forms, sometimes with mitotic induction (22). Similar observations have also been made in some fungi. Tsuda in 1979 reported the existence of a stimulating activity on the *in vitro* multiplication of mouse spleen lymphocytes by high doses of the lectin of *Flammulina velutipes* (74). The lectin isolated from *Lentinula edodes* was mitogenic towards murine splenic lymphocytes (116). More recently, it was shown that the lectin from *Boletus satanas* is mitogenic for human T lymphocytes with secretion of interleukins 1 a and 2 by mononucleate cells in culture (132). The L-fucose specific lectin isolated from *Aleuria aurantia* has mitogenic activity on mouse T lymphocytes (Thy-1^+ splenocytes and cortisone-resistant thymocytes) (133). The lectin from *Lentinula edodes* is a potent mitogen towards murine splenic lymphocytes (116).

Effect on pancreatic cells : The lectin from *Agaricus campestris* (PHA-A) and from *Agaricus bisporus* (PHA-B) can stimulate the production of

insulin and glucagon by rat pancreatic Langerhans cells and raise glucose consumption by adipocytes (134,135).

Effect on tumor cells : Certain fungal lectins showed inhibitory action on the *in vitro* growth of tumor cells. *Volvariella volvacea* lectin has a moderate effect on sarcoma-180 (136), and *Agaricus bisporus* lectin reversibly inhibited the proliferation of various lines of malignant epithelial cells, with no specific toxic effect (137). Moreover, some lectins, such as that of *Aleuria aurantia,* exert a selective toxic action on certain modified cell lines (138).

Structure and function

The amino acid sequence of *Xerocomus chrysenteron* lectin showed 69% and 64% homology with *Agaricus bisporus* and *Arthrobotrys oligospora* (Fig. 1.1). *Agrocybe aegerita* lectin showed structural similarity with galectins, the carbohydrate recognition domain (CRD) of which contains consensus sequence motif, which consists of His-44, Arg-48, Val-59, Asn-61, Trp-68, Glu-71 and Arg-73 (139). The deduced amino acid sequence of *Grifola frondosa* lectin showed 26.1% and 22.8% homology with jacalin related plant lectins from *Helianthus tuberosus* and *Parkia platycephala* (140).

Aleuria aurantia was the first fungal lectin, for which the crystal structure was solved. The crystal structure of the lectin complexed with fucose revealed that each monomer consists of a six-bladed β-propeller fold and a small antiparallel two-stranded β-sheet that plays a role in dimerization. Five fucose residues were located in binding pockets between the adjacent propeller blades (49). *Flammulina velutipes* lectin showed unique folds never before observed in lectins (141), and showed structural similarity to human fibronectin, lectin from *Xerocomus chrysenteron* (142) and *Agaricus bisporus* (143), resembling actinoporins, a family of pore-forming toxins from sea anemones. CGL2 from *Coprinus cinerea* showed fold similar to galectins (144), a large family of lectins from all classes of vertebrates (145). The lectin from *Laetiporus sulphureus* (146) showed resemblance with the ricin-B

domain, a trefoil-based fold observed in many lectins and carbohydrate-binding domains, and referred to as the (QxW)3 domain (147). This lectin assembles as a trimer, each monomer consisting of a ricin-B domain and an elongated domain with structural similarity to aerolysin, a bacterial β-pore-forming toxin. This domain could be directly involved in pore formation because the lectin displays hemolytic activity (148). The ricin-like domain displayed structural similarity to similar domains from plant toxins (ricin, abrin and mistletoe lectin), but was even more similar to the hemagglutinin component of bacterium *Clostridium botulinum* (149). Structural comparison of the (QxW)3 domains from mushroom toxin, clostridial hemagglutinin and ricin illustrated their high similarity. During evolution, this domain has been conserved, and often duplicated and/or combined with other domains. It has been identified in bacteria, fungi and plants, and also in sponge, insects and mammals, generally conserving its role of targeting a sugar-coated substrate (147).

A common six-bladed β-propeller fold was observed for bacterial lectin RSL from *Ralstonia solanacearum* (150) and fungal lectins AAL from *Aleuria aurantia* (151). The six-bladed β-propeller of lectin from *Aleuria aurantia* (AAL), has been crystallized with either five (49) or three bound fucose residues (152), suggesting that the binding sites, although all very similar in geometry, do not have the same affinity for ligands. The ligand-binding site of AAL is characterized by numerous hydrogen bonds to the side chains of polar amino acids and by strong hydrophobic interactions between aromatic residues. However, the lectin structures based on a β-propeller architecture are rare. The five- to eight-fold pseudo-symmetry offered by this architecture is very favorable for multivalent sugar binding. Site-directed mutagenesis carried out in *Aleuria aurantia* lectin showed that in β-2 mutant, out of five binding sites, substitution of Tyr^{26} (Site 1), Tyr^{79} (Site 2) and Tyr^{181} (Site 4) with Val resulted in loss of hemagglutinating activity, whereas mutation at Tyr^{133} (Site 3) and Tyr^{228} (Site 5) did not cause any loss of activity (153).

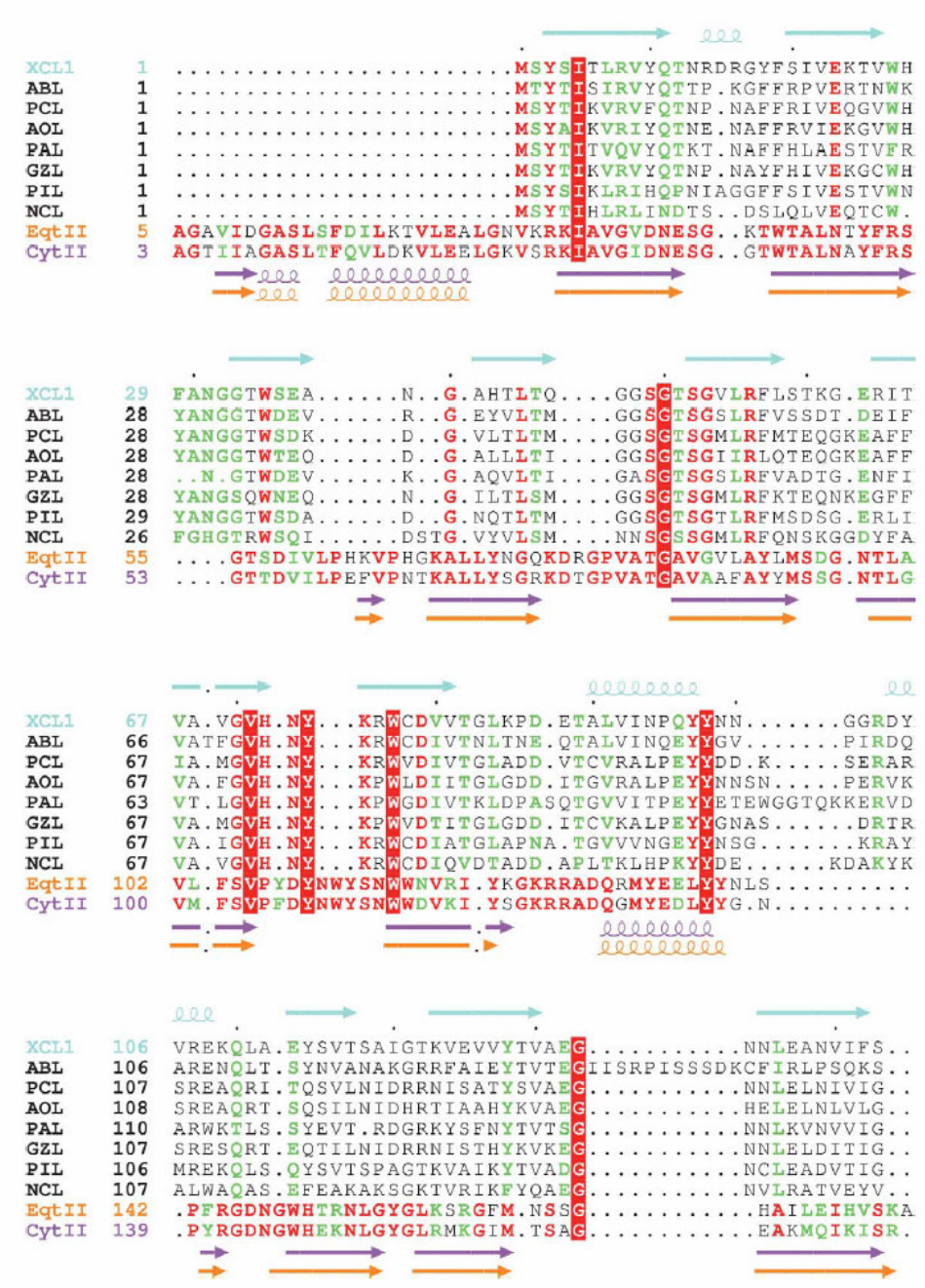

Fig 1.1. Sequence alignment of lectins from XCL- *Xerocomus chrysenteron* (154), ABL - *Agaricus bisporus* (143,155,156), PCL- *Pleurotus cornucopiae* (157,158), AOL- *Arthrobotrys oligospora* (159), PAL- *Podospora anserine* , GZL- *Gibberella zeae*, PIL - *Paxillus involutus* (160), NCL-*Neurospora crassa*. In each family, invariant and conserved residues are indicated by red type and green type, respectively. The secondary structure elements for XCL, equinatoxin II (EqtII) and sticholysin II (CytII) (same color code as the protein name) are indicated by coils for α-helices and arrows for β-strands. (Adapted from reference no. (142))

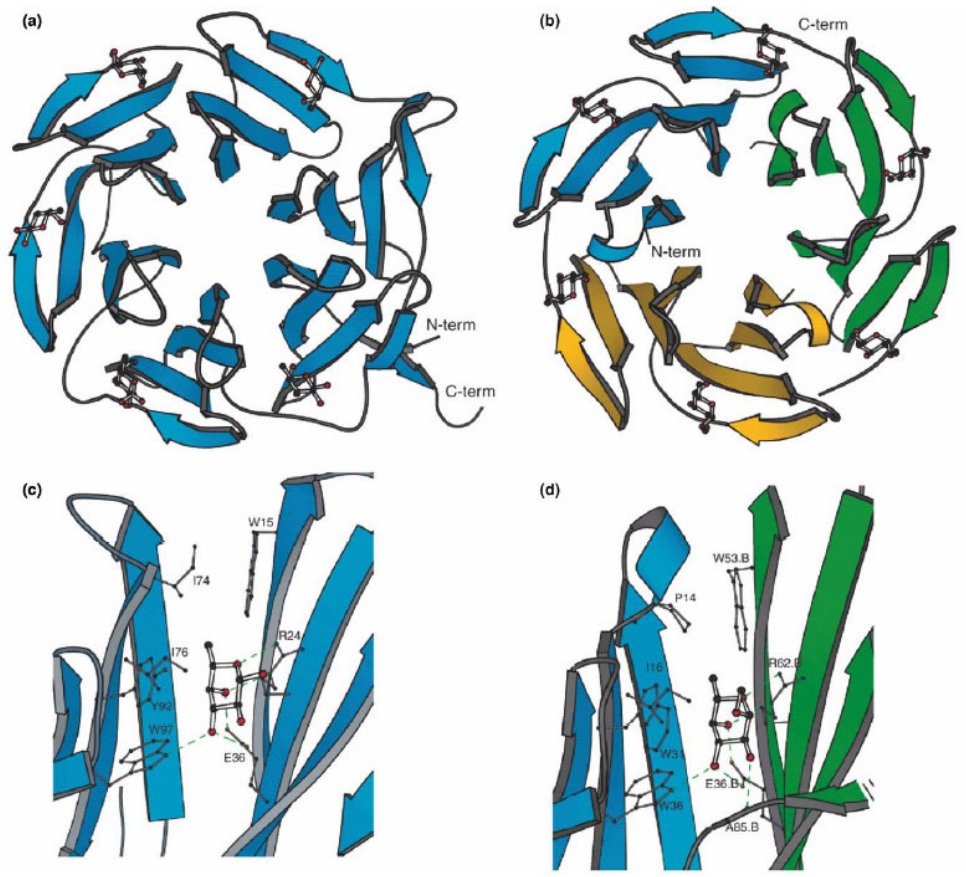

Fig. 1.2 : Crystallographic structures of the six-bladed β propellers formed by (a) one monomer of *A. aurantia,* AAL complexed with fucose (PDB code 1OFZ) and (b) a trimer of *R. solanacearum* RSL complexed with α-Me-fucoside (PDB code 2BT9). Carbohydrate-binding sites of (c) AAL and (d) RSL. Adapted from reference no. (161)

Chemical modification studies on lectin from *Ischnoderma resinosum*, showed involvement of lysine, carboxylate, arginine, tryptophan, histidine, cysteine and tyrosine in the binding activity of the lectin. Inhibitory sugar of lectin *viz.* lactulose could not protect modification against NBS mediated inactivation, whereas, substrate protection was observed in the case of glycine ethyl ester, cyclohexane-1,2-dione, ethoxyformic anhydride and NAI mediated

inactivation of the lectin (162). Tryptophan, carboxylate and tyrosine have been implicated in the binding activity of the lectin form *Hericium erinaceum* (163).

Biological role

Lectins, due to their ability to recognize different structures, constitute an important element of biological system. Their physiological role is connected with the identification of glycosylated structures at the level of cells, tissues and the whole organisms. Fungal lectin are reported to participate in the formation of primordia, creation of mycelium structures to facilitate, penetration of parasitic fungi into the host organism as well as mycorrhization (164,165).

1. Involvement in growth and morphogenesis

The lectins, *via* acting directly on carbohydrates by their specific binding sites, or indirectly by enzyme systems, possibly control depolymerization of stored material and intense movement of sugars, resulting initiation and often explosive growth of the sporomes. Lectin can repress the activity of the glycoprotein enzymes by binding them at their carbohydrate moieties. The release of simple sugars could thus redirect metabolism through competition for lectin binding (54).

A lectin may be involved in ensuring cohesion between hyphae during the development of the basidiome as seen in *Pleurotus cornucopiae* (166). However, the discovery in a strain of this species of a protein related to the lectin and regulated in the same way but not erythroagglutinant, makes the exact role of the lectin in this fungus uncertain. Either the lectin is not involved in the morphogenesis, or is but cannot be revealed by a hemagglutinating activity (167).

2. Involvement in dormancy

In some perennial fungus, variable lectin levels have been reported in different seasons, indicating possible role of lectin in the dormancy cycle, as in

Aegopodium podagraria (168) or *Eranthis hyemalis* (169). Studies done on *Helianthus tuberosus* established that lectins are directly involved in maintaining and ending dormancy (114,170).

3. Introducing morphological changes in the host

During their growth, many fungi build specialized organs that require mycelial aggregation. These organs, cords, fans of mycelium, strands, rhizoids *etc*. are found particularly in parasitic and wood-rotting species, which use these organs to force their way amongst the host cells. *Rigidoporus lignosus,* a parasite of many tropical trees infects roots by the vegetative mycelium, but propagates *via* the fans of mycelium. A lectin seems to be involved in the building of the latter structures, since it has been found in significant amounts only in the hyphae that are able to aggregate and in the fans of mycelium themselves (97,171). This lectin seems therefore to be a cell wall lectin involved in a recognition mechanism.

4. Molecular recognition during mycorrhization

Presence of lectins, with strict specificities, in fungal cells of ectomycorrhizal symbiosis, suggests that lectins might be involved in recognition between the tree and its symbiont. The hypothesis was further exemplified by study of lectins from three fungus *Lacterius deliciosus, L. deterrimus* and *L. salmonicolor,* though very close morphologically, are specifically associated with different trees *Pinus, Picea* and *Abies,* respectively. Three essential conditions were verified experimentally: (i) In spite of being present in closely related species the three lectin differ significantly in structures and their microspecificities towards oligosaccharides; (ii) It was demonstrated, by shedding from cell walls by enzyme degradation or location by means of polyclonal antibodies, that the lectin is present at the surface of cultured hyphae; (iii) The receptors, bore by the root cell surfaces of each of the conifers, were exclusively for the lectin of their own associated fungus (91).

Some of the molecules present in the soil can combine with fungal lectins and block their specific binding sites and in the rhizosphere, *e.g.* certain

phenolic acids that can modify carbohydrate receptors on the roots of the host trees, will prevent recognition and consequently mycorrhization (172).

Applications

The ability of lectin to interact with simple, aminated, acetylated, sialated and complex carbohydrates has been exploited for typing blood cells, carrier for chemotherapeutic agents, mitogens, fractionation of animal cells, or for studying cellular surfaces. They have also been used for the identification and differentiation of various microorganisms. They have also been successfully utilized as epidemiologic as well as taxonomic markers of specific microorganism (165,173).

1. Identification of different strains of microorganisms

Agglutinating extracts from several fungi have been used for the taxonomic study of protozoa in the genera *Crithidia, Blastocrithidia, Leishmania* and *Trypanosoma* (174-176). The fungal lectins from *Laccaria amethystea, Clitocybe geotropa* and *Pholiota squarrosa* have proved to be useful reagents in the taxonomy of rumen micromycetes and also utilized in monitoring changes in the cell walls occurring during their life cycle (177-179). The lectins of *Laccaria amethystea* and *Boletus edulis* have been used to study cysts and trophozoites of *Toxoplasma gondii* (180). Striking diversity of the carbohydrates on the surface of *Candida albicans* was observed. The lectin of *Russula nigricans* showed exclusive specificity towards *Candida albicans,* and can therefore be used in identification tests. It was also suggested that this lectin may also interact to structures involved in the pathogenecity of *Candida albicans* (130).

2. Use in food industry

The lectin of *Agaricus bisporus* was immobilized on magnetic beads and successfully used to remove *Listeria monocytogenes* bacteria added experimentally to milk (181).

3. To study role of glycoconjugates in embryo development

Fungal lectin have been used to study glycoconjugates, involved in the cellular interactions during the uro-genital morphogenesis in bird embryos, to understand the mechanisms responsible for the migration of germ cells. In birds, the primordial germ cells (PGCs), localized at the primitive line stage in the Swift crescent, subsequently migrate *via* the bloodstream towards future genital ridges. There are many arguments in favor of involvement of membrane glycoproteins in the mechanisms of recognition between PGCs and tissues during the migration. FITC-labeled lectins and NC-1/HNK-1 mono-clonal antibodies used in indirect fluorescence reactions bind to PGCs and afford ready visualization. The lectin of *Laetiporus sulphureus,* which recognizes N-acetyllactosamine residues is one of the most specific to this cell type (182-184).

4. Isolation and purification of serum glycoconjugates

Immobilization of lectin to an unreactive support also allows separation of glycoconjugates possessing lectin-specific glycan structures. The lectin isolated from *Aleuria aurantia* has been used for purification of fucosylated glycoproteins, such as H activity glycoconjugates from erythrocytes membrane (185), tumor antigens (186), Bence-Jones proteins (187), normal and pathologic human immunoglobulin-G (188) and brain glycoproteins (189,190).

5. In cancer research

Cancer cells exhibit altered specific properties such as loss of contact inhibition and ability to migrate and form metastases. Much work has been done to understand the modification occurring in membrane glycoconjugates, glycoproteins and glycolipids during cancer induction. Modification to glycoconjugates also includes the emergence of antigens that do not normally belong to the cells of the tissues concerned, or which are present in the membranes of the embryo cells and are then repressed in differentiated cells. These antigenic neo-expressions are generally linked to an impairment of

glycosyltransferase and glycosidase activities, and in some cases concern blood group antigens. A lectin from *Lacterius deliciosus,* which is specific to D-Galβ1→3D-GalNAc, can be used to follow the expression of the T antigen (Thomsen-Friedenreich antigen) in the same way as the lectin of peanut *(Arachis hypogaea)* (54). The residue, D-Galβ1→3D-GalNAc, in cancerous cells results from an improper sialylation in the glucid part of M or N blood group antigens and related structures (165).

6. Separation of cells

The property of lectin to differentiate surface can be used for the separation of certain cell types either from complex media or from a mixture of different cell categories by exploiting difference in surface sugars. The cell sorting, using lectin was first attempted by Nowell (22) to separate red cells from white in human blood using phytohemagglutinins that led to the discovery of the lymphocyte-modifying properties of bean lectin. The lectin is immobilized on inert supports, in columns or magnetic bead followed by passing of the mixture over beads. Another approach is to exploit the toxic properties of lectins, whereby binding to specific sites causes cell death, only cells devoid of such sites surviving the treatment (165).

7. Sorting of mutant and tumor cells

This technique involves toxic property of the lectins. In case of non-toxic lectins, chain A of ricin (effectomer) or diphtheria toxin can be attached to them to render toxicity. Another approach is to treat the cells, after lectin binding, with an anti-lectin antibody and complement. The cell sorting has been applied in two separate areas: the selection of modified cell lines and the sorting of cells derived from artificially induced mutations. The selection of such mutant cells involves alteration in glycan chain of the surface glycoconjugates. Such alterations are caused by mutations affecting enzymes, such as glycosyltransferases and glycosidases or enzymes involved in the synthesis of intermediates such as nucleotide sugars or lipid-bound sugars. In another approach, from ‘wild’ phenotype (lectin sensitive) to resistant lectin

are created. In this way it is possible to select a wide range of mutants using a large number of lectins, also identical mutants can be sorted using lectins with different specificities. The main value of this selection is to understand structure-activity relations, since non-binding of the lectin implies that an enzyme protein is missing or functionally impaired (54).

The other area of application is the detection, in a population of cells modified by oncogenesis, of lines displaying resistance to particular lectins. As above, this resistance is linked to alterations to surface glycans, which very often form the essential structures of receptor molecules or are involved in mechanisms of recognition between the cell and its environment. In this way it is possible to investigate how modifications to particular glycan structures affects the tumor-forming capacity of the cells and their metastatic potency (165).

The lectin of *Aleuria aurantia* has been used to study correlations between lectin-resistance and growth characteristics on cell lines from Lewis-type (LL2) mouse lung cancers (106,138,191,192) and line Mm B16 of mouse melanoma (192).

FEW IMPORTANT FUNGAL LECTINS

Lectins from Mushrooms

The mushroom lectins have been localized on different parts as the caps, stipes and mycelia. The lectin content varies in the mushroom and depends on carpophore age, and the time and place of harvest. Lectins have been reported in several edible and non-edible mushrooms. In mushrooms, lectins probably play an important role in dormancy, growth, morphogenesis, morphological changes consequent on parasitic infection and molecular recognition during the early stages of mycorrhization (165). A detail survey of mushroom lectin is documented in the review articles by Kawagishi (33), Guillot and Konska (165) and Wang *et al.* (193).

Agaricus : Several species of mushroom *Agaricus* were found to contain lectins. It appears that *A. bisporus* produces more than one lectin (29). So far, two lectins PHA and PHB have been reported from the mushroom. The lectin are tetrameric and all have molecular mass of 64 kDa and 58 kDa (104). Hemagglutinating activity of the lectins can be inhibited by bovine and porcine submaxillary mucins (104). Both the lectins have similar specificities for cell surface carbohydrate receptors and have been purified by chromatography on DEAE-cellulose, Sephadex G-150, phosphocellulose and Sephadex G-150. PHA and PHB could be separated by phosphocellulose column chromatography. PHA is a glycoprotein with 2.5% carbohydrate. The lectins have been shown to stimulate the release of both insulin and glucagon from the pancreatic islets of Langerhans (134,135). The *A. bisporus* lectin has been reported to have an antiproliferative activity toward various tumor cell lines (137). The binding properties of ABA was investigated in detail by enzyme-linked lectinosorbent assay and lectin-glycan inhibition assay. The results indicated that polyvalent Galβ1→ related glycotopes, GalNAcα1→Ser/Thr (Tn), and their cryptoforms, were the most potent ligand for the lectin (194). Lectin from *A. blazei* has a molecular mass of 64 kDa and composed of four subunits each with a molecular mass of

16 kDa same as *A. bisporus* and it was glycosylated. Its hemagglutinating activity was inhibited by glycoproteins *viz.* asialo bovine submaxillary mucin, asialo-fetuin and fetuin; and monosaccharides as *N*-acetyl-D-galactosamine (85).

A lectin reported from *A. campestris* has similar molecular mass and subunit as lectin from *A. bisporus* and *A. blazei*. But the lectin from *A. campestris* was more thermostable (75,195). *A. edulis* also produced two lectin as reported by Eifler, both the lectins were different in molecular mass and carbohydrate specificity (196). One of them was tetrameric with a molecular mass of 60 kDa while the other was dimeric with a molecular mass of 32 kDa. The lectins showed very high thermostability and could withstand high concentration of urea and extreme pH. One of the lectins has complex specificity and its hemagglutinating activity can not be inhibited by common simple sugars.

Lectin has been isolated from *A. cylindracea* by Yagi *et al.* (197) and Wang *et al.* (198) The lectin purified by Yagi *et al.* (197) is homodimeric with a blocked N-terminus while that of Wang *et al.* (198) is heterodimeric with distinct N-terminal sequences for the two subunits. Both the lectins were sialic acid specific. Lactose was a poor inhibitor of the hemagglutinating activity of the lectin purified by Yagi *et al.* (197) but effectively counteracted the activity of the lectin purified by Wang *et al.* (198).

The *A. cylindracea* lectin exhibited mitogenic response. A six-fold increase was observed in mitogenic response when mouse splenocytes were incubated with 2 μM *A. cylindracea* lectin. A dose-dependent increase in mitogenic response could be observed when the lectin concentration was elevated from 0.5 to 1 μM and then to 2 μM. When the lectin concentration was further increased to 4 and 8 μM there was a decline in mitogenic response. The mitogenic responses at these concentrations were, however, over 200% higher than the control value (198).

Armillaria luteo-virens : From the dried fruiting bodies of the mushroom *A. luteo-virens*, a dimeric lectin with a molecular mass of 29.4 kDa was isolated. Its hemagglutinating activity could be inhibited only by polysaccharide inulin but not by simple sugars. The activity was stable up to 70 °C but was sensitive to high and low pH. The striking feature was the inhibition of activity by salts including $FeCl_3$, $AlCl_3$, and $ZnCl_2$ but not by $MgCl_2$, $MnCl_2$, and $CaCl_2$. The lectin stimulated mitogenic response of mouse splenocytes. Proliferation of tumor cells including MBL2 cells, HeLa cells, and L1210 cells was inhibited by the lectin. However, proliferation of HepG2 cells was not affected (98).

Amanita pantherina : A dimeric lectin with a molecular mass of 43 kDa was purified from *A. pantherina* by hydrophobic chromatography on Butyl-Toyoperls, affinity chromatography on submaxillary mucin-Toyopearl and gel filtration on Superose-12. Its hemagglutinating activity was inhibited by a number of oligosaccharides, bovine submaxillary mucin and asialo-bovine submaxillary mucin. The lectin was active in the pH range 4-9.5 and a temperature below 40 ºC. The lectin did not show requirement of any metal ion for its activity. It was found to be a glycoprotein with carbohydrate content of 4.3 % and characterized by high content of Gly, Glx, Asx, and Ser, and low content of Met, Lys, Tyr. The N-terminal sequence of the lectin was Ile-Phe-Ala-Val-Gly-Glu-Thr-Gln-Gly-Glu (199).

Agrocybe cylindracea : An antifungal peptide, designated as agrocybin, with a molecular mass of 9 kDa was isolated from fresh fruiting bodies of the mushroom *A. cylindracea*. It showed antifungal activity against several fungal species but not antibacterial activity. The activity of HIV-1 reverse transcriptase was attenuated in the presence of agrocybin. It exhibited weaker mitogenic activity than Con-A on isolated murine splenocytes, but was devoid of antiproliferative activity on HepG2 (hepatoma) cells (200).

Boletus satanas : The lectin of *B. satanas,* designated as bolesatine was found to be a toxic glycoprotein with hemagglutinating activity. It was purified by ammonium sulfate precipitation and anionic exchange chromatography on FPLC and found to have a molecular mass of 63 kDa. It exhibited mitogenic activity toward lymphocytes and elicited the release of interleukins and tumor necrosis factor from mononuclear cell cultures (201). It inhibited DNA and protein synthesis in Maidn Darby canine kidney cells with an IC_{50} of 0.62 and 0.14 μM respectively and the inhibitory effects could be reversed by addition of galactose (30). These biological activities of bolesatine might contribute to the toxicity of *B. satanas.*

Coprinopsis cinerea : Two different dimeric lectins (CGL1 and CGL2) were purified by ion exchange and affinity chromatography on lactosyl-Sepharose from *C. cinerea* (formerly *Coprinus cinereus*) during fruiting body formation. The lectins were found to be structurally similar to galectins, a group of galactose specific animal lectins, and demonstrated 83 % sequence homology and conservation of all key residues present in galectins. They are different from most galectins in the absence of cysteine residues and N-terminal post-translational modification (31).

The role of these lectins in tissue development was studied by Walser *et al.* (108). The developmentally regulated ligands for galectins were co-localized with galectin expression, in the veil surrounding the developing primordium and the outer cells of the young stipe. In addition, galectin ligand were observed in the hymenium. The subcellular localization of the galectin ligands suggested these to be present in cellular compartment distinct from galectin transport. The sensitivity of the *in situ* interactions with exogenous galectins towards detergents and organic solvents inferred that these ligands were lipid-borne. Accordingly, lipid fractions from primordia were shown to contain galectin-binding compounds. Based on these observations it was hypothesized that β-galactoside-containing lipids

(basidiolipids) found in mushrooms are physiological ligand for the galectins in *C. cinerea* (108).

The crystal structure of CGL2 was solved and showed fold similar to vertebrate galectins. The 2.1 Å crystal structure of CGL2 and five substrate complexes revealed that this prototype galectin achieves increased substrate specificity by accommodating substituted oligosaccharides of the mammalian blood group A/B type in an extended binding cleft. Kinetic studies on wild-type and mutant CGL2 proteins demonstrated that the tetrameric organization is essential for functionality. The geometric constraints due to orthogonal orientation of the four binding sites have important consequences on substrate binding and selectivity (145).

Flammulina velutipes : *F. velutipes* exhibited some peculiar properties compared to other mushroom lectins. It was found to be a heterodimer of 12 kDa and 8 kDa subunits. The lectin was purified by zinc acetate treatment and CM-cellulose column chromatography. The lectin was devoid of carbohydrate, half-cysteine, methionine, and histidine. Upon gel filtration on Sepharose 6B in the presence of 6 M guanidine-HCl the subunits of the lectin could be separated. Only the larger subunit of the lectin had hemagglutinating activity; and the hemagglutinating activity was not inhibited by any of the simple sugars. The lectin supposedly has affinity for more complex carbohydrate structures on the cell surface. The larger subunit also promoted proliferation of mouse splenocytes and elicited mitogenic activity (74).

Ganoderma capense : The lectin showed similarity to fungal immunomodulatory proteins and very high temperature stability (stable at 100 °C for 60 min). Its mitogenic activity was more potent than Con-A towards mouse splenocytes and showed antiproliferative activity towards leukemia (L 1210 and M1) cells hepatoma (HepG2) cells. However, no antifungal and HIV-1 reverse transcriptase activity was observed (32).

Grifola frondosa : An *N*-acetylgalactosamine specific lectin was purified by affinity chromatography on acid-treated Sepharose CL-4B and subsequently on GalNAc-Toyopearl from fruiting bodies of *G. frondosa.* Its molecular mass determined by gel filtration using various buffers and matrices, ranges from 30 to 52 kDa. On the other hand, SDS-PAGE of the lectin in the presence or absence of 2-mercaptoethanol showed three major bands with molecular mass of 33, 66 and 100 kDa and a faint band of 65 kDa. The lectin contained high amount of acidic and hydroxyl amino acids, glycine and alanine, and low amount of methionine, phenylalanine, isoleucine and histidine residues. *G. frondosa* lectin was glycosylated and contained 3.3 % neutral sugar. Dialysis of the lectin with chelating agent, divalent metals including $CaCl_2$, $MgCl_2$, $ZnCl_2$ or $MnCl_2$ did not alter the hemagglutinating activity of the lectin. The lectin exhibited cytotoxic activity against the HeLa cells, the minimum concentration required to induce death of all the cells was 25 μg/ml. The toxicity could be inhibited by pre-incubating the lectin with the haptenic sugar *N*-acetylgalactosamine (33).

Ganoderma lucidum : An immunomodulatory protein was isolated from *G. lucidum* mycelia having molecular mass of 12.4 kDa. The protein designated as LZ-8 was a small protein of 110 amino acid residues. The N-terminal was found to be blocked by *N*-acetylation. It was glycosylated. The lectin activity was observed with ovine but not with human erythrocytes (202). It displayed many biological activities characteristic of lectins including mitogenic and hemagglutinating activities. Other biological activities of LZ-8 included mitogenic activity on mouse splenocytes and human peripheral lymphocytes, and suppression of anaphylaxis induced by bovine serum albumin in CFW mice (203,204).

G. lucidum was also found to produce a lectin with a molecular mass of 18 kDa. It was purified by anion exchange chromatography and affinity chromatography on asialobovine submaxillary mucin-Toyopearl. Another lectin was isolated from the fruiting bodies by the same procedure (79).

The two lectins isolated by Kawagishi *et al*. (79) were distinctly different from LZ-8 reported by Tanaka *et al*. (202), in their molecular masses and sugar-binding activities.

Hericium erinaceum : The *H. erinaceum* lectin was a heterotetramer with molecular mass of 54 kDa as determined by gel filtration. It has two different subunits with molecular mass of 15 and 16 kDa. It contained substantial amount of glycine, glutamic acid or glutamine, serine, alanine and a small amount of tyrosine, histidine and methionine. It was a glycoprotein with 1.5 % carbohydrate and showed specificity towards neuraminic acid and especially with *N*-glycolylneuraminic acid. Considerable thermal stability was observed in the lectin as it was stable upto 70° C. Its hemagglutinating activity was not affected by demetalization with EDTA or by addition of $CaCl_2$, $MgCl_2$, $ZnCl_2$ or $MnCl_2$ (34).

Ischnoderma resinosum : From the fruiting body of *I. resinosum* a lectin was purified by affinity chromatography on Sepharose-4B. The lectin is made up of two identical subunits, each with a molecular mass of 16 kDa. It contained substantial amounts of acidic and hydroxy amino acids, glycine, valine and leucine, but minute quantities of methionine, histidine and arginine. The hemagglutinating activity of the lectin can be inhibited by Methyl β-galactoside, L-Fucose and L-arabinose (85).

Laetiporus sulphureus : The parasitic mushroom *L. sulphureus* produced a lectin in carpophore, and was purified by affinity chromatography on Sepharose. The lectin also showed hemolytic activity. Some microheterogeneity was observed in isoelectric focusing although the HPLC profile exhibited homogeneity. Its molecular mass, as determined by gel filtration and also by electrophoresis under non-denaturing conditions, was around 190 kDa. The lectin was tetrameric, consisting of two distinct types of subunits (with molecular mass of 60 and 36 kDa

respectively). It contained threonine, valine, and glycine residues in abundance, while aspartic acid and sulfur containing amino acids occurred in extremely small quantities. No carbohydrate was detected. Hapten inhibition assays indicated that lectin is specific for *N*-acetylglucosamine residues and the same site of the lectin molecule contributes to both hemagglutinating and hemolytic activities (115).

Lacterius deterrimus : A lectin from carpophores of *L. deterrimus*, a specific symbiont of spruce, was isolated using steps including, affinity, hydroxylapatite, and gel filtration chromatographic techniques. It showed molecular mass of 37 kDa in gel filtration. Although it was apparently homogeneous in HPLC gel filtration, isoelectric focusing showed microheterogeneity with a main band in the zone near pH 6.5. Amino acid analysis showed that the lectin contained high amount of glycine and methionine. Carbohydrate could not be detected in the lectin. Hapten inhibition assays indicated that *L. deterrimus* lectin was most specific for β-D-galactosyl(1-3)-D-*N*-acetylgalactosamine residues. The lectin was produced by cultured mycelia, and could be localized by immuno-fluorescence in the cell wall. Receptors for the lectin were localized on the root hairs of axenically grown spruce seedlings. This inferred a role of the fungal lectin in recognition and specificity during the early stages of mycorrhiza formation (91).

Lyophyllum shimeiji : A hemagglutinin, with a molecular mass of 30 kDa was isolated from fresh fruiting bodies of the edible mushroom *L. shimeiji*. However, the hemagglutinating activity could not be inhibited by simple sugars and glycoproteins. The hemagglutinin exhibited a novel N-terminal sequence not found in any lectin and hemagglutinin (205).

Pleurotus ostreatus : A dimeric lectin, from fresh fruiting bodies of the edible mushroom *P. ostreatus,* was purified by ion-exchange chromatography. It was composed of two subunits with a molecular mass of 40 and 41 kDa,

respectively, and demonstrated similarity in N-terminal sequence to each other and to *Aleuria aurantia* lectin. The lectin was sensitive to extreme pH and heat. Mellibiose, lactose, D-galactose, α-methyl-D-galactopyranoside, N-acetylneuraminic acid, raffinose, and inulin were capable of inhibiting its hemagglutinating activity and mellibiose was the most potent inhibitor. The lectin exerted potent antitumor activity in mice, bearing sarcoma S-180 and hepatoma H-22 cells. Survival in these mice was prolonged and body weight increase reduced after lectin treatment (206).

The activation of an endogenous phosphatase by the lectin was observed for ADP and ATP. The lectin was also found to have α-galactosidase activity, the enzyme activity and carbohydrate binding property was not due to same site since the lectin accepts both α- and β-glycosides whereas the enzyme activity was restricted to the α-anomer only. Moreover, the α-galactosidase activity was inhibited by α-galactose but not by β-galactose. Therefore, lectin and enzymatic activities were either properties of two tightly associated proteins, or of just one molecule (92). Crystals of *P. ostreatus* lectin were grown by the hanging-drop technique using ammonium sulfate as the precipitant at 293 °K. Over a period of between two and three weeks, crystals of hexagonal bipyramidal morphology grew to maximum dimensions of $0.2 \times 0.2 \times 0.5$ mm (207).

Pleurotus tuber-regium : The fresh sclerotia of the edible mushroom *P. tuber-regium* showed presence of an N-acetylglucosamine-binding lectin, with a molecular mass of 32 kDa. Its N-terminal sequence showed some similarity to that of *Agaricus bisporus* lectin. The lectin exhibited hemagglutinating activity toward trypsinized rabbit erythrocytes but not toward untrypsinized rabbit erythrocytes (208).

Polyporus adusta : The lectin from *P. adusta* was purified by ion-exchange chromatography on DEAE-cellulose, Q-Sepharose and CM-Sepharose.

The hemagglutinating activity of the lectin was inhibited by turanose and several other carbohydrates as D(+)-mellibiose, D-fructose, L-arabinose and glucose. Hemagglutinating activity of the lectin showed 100% increase in the presence of $FeCl_3$ (10 mM). The lectin also showed antiproliferative activity towards tumor cell lines and mitogenic activity towards splenocytes (55).

Peziza sylvestris : The lectin isolated from *P. sylvestris* was the first arabinose specific fungal lectin with mitogenic activity towards splenocytes. It was a single chained protein with a molecular mass of 20 kDa. The N-terminal sequence showed only slight resemblance to other mushroom lectins. Its hemagglutinating activity was inhibited by arabinose, but not by a large variety of other carbohydrates (82).

Schizophyllum commune : A homodimeric lactose-binding lectin with a molecular mass of 64 kDa was isolated from fresh fruiting bodies of the split gill mushroom *S. commune*. The N-terminal sequence of the lectin showed similarity to a part of the sequence of the cell division protein from *Gleobacter violaceus*. The hemagglutinating activity of the lectin was stable at temperatures up to 40 °C, and in concentrations of NaOH and HCl solution up to 125 and 25 mM, respectively. The lectin exhibited potent mitogenic activity toward mouse splenocytes, antiproliferative activity toward tumor cell lines, and inhibitory activity toward HIV-1 reverse transcriptase (209).

Tricholoma mongolicum : Two lectins were purified from edible mushroom *T. mongolicum* and designated as TML-1 and TML-2. The lectins were homodimers, with subunit molecular mass of 17.5 kDa. Hydroxyproline was present in the lectins but no carbohydrate was detected. The hemagglutinating activities of the lectins were inhibited by α-lactose, β-lactose, lactose, *N*-acetyl-D-galactosamine and D-galactose, and were abolished in extreme pH values. EDTA, $CaCl_2$, $MgCl_2$ did not affect the

hemagglutinating activity of the lectin, reduction of the activities occurred, however, after addition of $ZnCl_2$ or $MnCl_2$. Thermal stability between 10° C to 80° C was observed. The two lectins manifested antiproliferative activity against mouse monocyte-macrophage cells and mouse mastocytoma cells *in vitro* (35). Both of them were able to inhibit the growth of implanted sarcoma 180 cells by 68.84% and 92.39% respectively. The growth of tumor cells in the mouse peritoneal cavity was also inhibited by the two lectins with TML-2 expressing a greater potency (210).

These lectins stimulated the production of nitrite ions by macrophages in normal and tumor-bearing mice. Both lectins exhibited negligible mitogenic activity towards T-cells or splenocytes from normal mice *in vitro* when compared with Con-A. Similarly, T-cells from sarcoma-bearing mice treated with the lectins did not exhibit a mitogenic response different from that of the control. However, T-cells from normal lectin-treated mice demonstrated a reduced mitogenic response when compared with the control. In mice, the growth of sarcoma 180 cells in the peritoneal cavity was inhibited and the life-span was prolonged by the two lectins (211).

Volvariella volvacea : A lectin (VAG) was extracted from fruiting bodies of *V. volvacea* by ammonium sulfate fractionation, DEAE-C-52 and CM-C-52 column chromatography. The molecular mass was estimated to be 26 kDa comprising two non-identical subunits. The lectin was rich in aspartic acid, glutamic acid, tyrosine and leucine but lacking free-cysteine, methionine and histidine. Its hemagglutinating activity was preserved even after incubation at 80 °C for 2 h. The hemagglutinating activity of the lectin was not inhibited by any of the common simple sugars. It is likely that the lectin has an affinity for complex carbohydrate structures on the cell surface. The *V. volvacea* lectin showed toxicity, its LD_{50} in mice was 17.5 mg/kg body weight. It has a mild retarding effect on the growth of tumor cells (136).

One more lectin (VVL) was isolated from the fungus by She (212). The lectin was purified from the fruiting bodies as well as cultured mycelia. It was a homodimeric protein with a molecular mass of 32 kDa as demonstrated by gel filtration and SDS-PAGE. Its hemagglutinating activity was inhibited by thyroglobulin but not by simple carbohydrates such as monomeric or dimeric sugars. The immunomodulatory activity of the lectin was demonstrated by its potent stimulatory activity toward murine splenic lymphocytes. It was also found to markedly enhance the transcriptional expression of interleukin-2 and interferon-gamma by reverse transcriptase-polymerase chain reaction. As revealed by its N-terminal amino acid sequence, it was distinct from other immunomodulatory proteins previously reported (213) and VAG (136) from the same fungus.

Xerocomus spadiceus : The lectin was capable of eliciting an approximately 4 fold stimulation of mitogenic response in murine splenocytes. The hemagglutinating activity was stable up to 60 °C. The hemagglutinating activity was increased in the presence of $AlCl_3$ or $ZnCl_2$ and reduced in the presence of HCl and NaOH. Among the large number of carbohydrates tested, only inulin was able to inhibit the hemagglutinating activity of the lectin (96).

Lectins from other fungi

Arthrobotrys oligospora : *A. oligospora* a nematophagous fungus has a surface lectin, mediating the capture of nematodes. It was a dimeric glycoprotein with a molecular mass of 36 kDa and an isoelectric point of pH 6.5. Its hemagglutinating activity can be inhibited by glycoproteins like fetuin and mucin but not by monosaccharides or disaccharides. The activity was higher in the alkaline range (86).

Dictyostellium discoideum : A lectin, designated as discoidin-I, was purified from slime mold *Dictyostellium discoideum.* The lectin was found to play

important role in intercellular adhesion during development of the slime mould (214).

Aspergillus fumigatus : The lectin showed apparent molecular mass of 32 kDa, and specificity for sialic acid. It was speculated that the lectin may contribute to the attachment of conidia to the extracellular matrix components through the recognition of the numerous terminal sialic acid residues of their carbohydrate chains (52).

Kluyveromyces bulgaricus : Two galactose-specific lectins, with molecular masses of 38 and 150 kDa, respectively, have been isolated from the yeast *K. bulgaricus.* The 38 kDa lectin was found to associate at higher concentration and form the octameric 150 kDa lectin. Both the lectins were glycoproteins, which agglutinate human erythrocytes and flocculate EDTA-treated *K. bulgaricus* cells. The cell wall phosphopeptidomannan was found to play a role as a ligand and a potential physiological receptor of the lectins (215).

Sclerotium rolfsii : A lectin was isolated from phytopathogenic fungus *S. rolfsii*. It was a homodimeric lectin of 34 kDa, which monomerize at acidic pH 4.3 to give 17 kDa subunits. The lectin agglutinated asialo human ABO as well as rabbit erythrocytes. Its hemagglutination activity was strongly inhibited by fetuin but not by mono- and oligosaccharides. Its binding property was studied by enzyme linked lectinosorbent assay (ELLSA) and by inhibition of lectin-glycan interaction. Among glycoproteins tested for binding, it reacted strongly with GalNAcα1$\rightarrow$Ser/Thr (Tn) and/or Galβ1$\rightarrow$3GalNacα1$\rightarrow$(T_α) containing glycoproteins: human T_α and Tn glycophorin, asialo-BSM, asialo-PSM, and asialofetuin, but its reactivity towards sialated glycoprotein was reduced significantly (121).

Rhizoctonia solani : *Rhizoctonia solani* lectin (RSA) was a homodimeric protein, composed of noncovalently associated 13 kDa monomers. The

lectin was found to have pI above pH 9 and it was composed of mainly β-sheets. The N-terminal sequence showed homology with subdomain of ricin-B. It exhibited specificity towards N-acetyl-galactosamine, and preferentially agglutinated human type A over type B and O, erythrocytes (95). It demonstrated specificity towards Gal and GalNAc whereby the hydroxyls at the C3', C4', and C6' position of the pyranose ring played a key role in the interaction with simple sugars. The carbohydrate-binding site of RSA apparently accommodates only a single sugar unit (94).

Beauveria bassiana : *The B. bassiana* lectin (BBL) was a 15 kDa glycoprotein rich in hydrophobic amino acid, without detectable amount of methionine. It contained 12.6 % of carbohydrates including galactose and mannose. It did not show blood group specificity and metal ion requirement. The hemagglutination caused by the lectin was inhibited by α-lactose (Galβ1→4Glcα), but not by β lactose (Galβ1→4Glcβ). In direct ELISA the lectin preferentially reacted with some glycoproteins carrying O-linked sugar structure Galβ1→3GalNAc, *e.g.* strongly with human glycophorin-A, and weaker with mouse glycophorin, fetuin, IgA and ovine submaxillary mucin. However, it did not react with N-glycoproteins *viz.* α_1-acid glycophorin, haptoglobin and fibronectin. Moreover, the interaction of BBL with highly sialated preparations of glycoproteins was weaker than with asialo forms (76).

Sclerotinia sclerotiorum : The lectin from mycelium and sclerotes of the phytopathogenic fungus *S. sclerotiorum* was a homodimer of two identical non-covalently bound subunits of 16 kDa. CD spectra analysis revealed that the *S. sclerotiorum* agglutinin (SSA) contained predominantly β-sheet structures. SSA exhibited specificity towards GalNAc whereby the hydroxyl at position 4 and 6 of the pyranose ring played a key role in the interaction with simple sugars. The carbohydrate-binding site of SSA can

also accommodate disaccharides. The N-terminal sequence of SSA showed no significant similarity with any other protein except a lectin from the fungus *Ciborinia camelliae.* A comparison of SSA and some other lectin from Sclerotiniaceae indicated that the Sclerotiniaceae lectins form a homogenous family of fungal lectins (51).

Rhizopus stolonifer : The lectin from *R. stolonifer* (RSL) was purified by affinity chromatography with porcine stomach mucin-Sepharose. SDS-PAGE and mass spectral analysis showed that RSL is 4.5 kDa protein, whereas gel filtration indicated a mass of 28 kDa. This indicated the lectin was a hexamer of noncovalently associated RSL monomers. RSL activity was very stable since it was insensitive to heat treatment at 70 °C for 10 min. Analysis of RSL binding specificity by both microtitre plate and precipitation assays showed that N-glycans with L-fucose linked to the reducing terminal GlcNAc residues were the most potent inhibitors, whereas N-glycans without α(1→6) linked fucose residues were ~100 fold weaker inhibitors. Oligosaccharides with α(1→2, 1→3, and 1→4) linkages showed no inhibition of binding in these assay. In a mirror resonance biosensor assay, high affinity binding was observed between RSL and the glycopeptide of bovine-γ-globulin, which has N-glycan with α(1→6)-linked fucose residues. However, RSL showed only a weak interaction with the glycopeptide of quail ovomucoid, which lacks fucose residues (77).

Present Investigation

Endophytic fungi, which form non-pathogenic colonies in the host plant without development of pathogenic symptoms are useful to the host since it can enhance plant growth (216), limit pathogen damage (217) and help in mineral absorption. Sometimes they can become pathogenic and hence they are considered between true symbionts and benign parasites. Studies of endophytic fungi indicate that they occupy a unique ecological niche, and are thought to influence plant distribution, ecology, physiology, and biochemistry (218). Endophytic fungi are one of the largely untapped resources, which can have economically important applications for production of enzymes, medicines and biological control agents. In view of the importance of fungal lectins from a basic as well as applied aspects attempts were made to look for endophytic fungal cultures for the presence of lectin. Screening of several endophytic fungal strains showed that an isolate of *Fusarium* sp. (LR11), produced high hemagglutinating activity when grown on MGYP medium. Hence the present investigation was carried out to purify and characterize this lectin to understand its structure-function correlations. Its binding studies were carried out with different ligands using spectrofluorimetry and SPR, which provides vital informations regarding subtle nature of specificity. Conformational stability of the lectin was also determined for different chemical and physical chaotropes.

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CHAPTER : 2

PURIFICATION AND CHARACTERIZATION OF *FUSARIUM* LECTIN

SUMMARY

A lectin from the mycelial extract of an endophytic strain of *Fusarium* sp. was purified to homogeneity by successive and repeated chromatography on Phenyl-Sepharose followed by gel filtration with an overall yield of 26%. The molecular mass of the lectin determined by gel filtration is 26 kDa and it is made up of two identical subunits of 13 kDa. It is a basic protein with a pI of 8.7. The purified lectin is a glycoprotein and contains 3.9% carbohydrate. It exhibited high pH stability and temperature stability. The partial N-terminal sequence did not show similarity with any known lectin. Purified lectin agglutinated neuraminidase/pronase treated human erythrocytes (A, B and O), with very low titre values. Its hemagglutinating activity was inhibited by glycoproteins containing N-linked or O-linked glycans. Chemical modification studies suggested the involvement of tyrosine in hemagglutinating activity.

INTRODUCTION

Lectins are non-immunogenic proteins or glycoproteins that selectively bind carbohydrates without processing them enzymatically (1). They are widespread in origin and have been isolated from animals, plants, bacteria, viruses and fungi. Although extensive studies have been carried out on plant and animal lectins (2,3), very little information is available on lectins from fungal sources (4,5). However, over the past decade fungal lectins have attracted increased attention due to their antitumor, antiproliferative and immunomodulatory activities (6-8).

Endophytic fungi, which colonize inside the host plant in an asymptomatic way, are between benign parasites and true symbionts. Screening of several endophytic fungal cultures revealed that the mycelial extract of *Fusarium* sp. (LR11/NCIM1330) produces high levels of hemagglutinating activity with complex sugar specificity. The present Chapter describes the purification and characterization of a lectin from mycelia of *Fusarium*.

MATERIALS

N-Acetylimidazole, 2,4,6-trinitrobenzenesulphonic acid, phenylglyoxal, diethylpyrocarbonate, phenylmethylsulfonyl fIuoride, N-bromosuccinimide, 5,5' dithiobis-(2-nitrobenzoic acid), sodium borohydride, hydroxylamine hydrochloride, galactose, galactosamine, 2-deoxygalactose, L-fucose, glucose, mannose, methyl-α-D-galactose, methyl-β-D-galactose, mellibiose, lactose, Phenyl-Sepharose CL-4B, pronase-E, all glycoproteins, gums and the molecular weight markers (Sigma Chemical Co. St. Louis, U.S.A); Sephadex G-25 and Sephacryl S-200 (Amersham Bioscience, Uppasala, Sweden); trypsin and neuraminidase (Sisco Research Laboratories, Mumbai, India) and dextrose, peptone, malt extract, agar and yeast extract (HiMedia Laboratories Pvt. Ltd, Mumbai, India), were used. All other reagents were of analytical grade.

METHODS

Erythrocyte preparation

Human erythrocytes of A, B and O blood groups were washed 5 to 6 times with 20 mM Tris-HCl buffer pH 7.2 containing 150 mM NaCl. A 3% (v/v) suspension of the erythrocytes in the above buffer was treated with different enzymes (pronase 0.05%, trypsin 0.05% and neuraminidase 0.1 U/ml) at 37 ºC for 1 h, washed 3 times with the same buffer and used for further studies.

Hemagglutination assays

Hemagglutination assays were performed in standard microtitre plates by the two-fold serial dilution method. A 50 µl aliquot of the erythrocytes suspension was mixed with 50 µl of serially diluted lectin and agglutination was examined visually after incubation for one hour. A unit of hemagglutination activity (U) is expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination. The specific activity of the lectin is defined as the number of hemagglutination units/mg of the protein.

Hemagglutination inhibition assays

Hemagglutination inhibition assays were performed similarly, except that serially diluted sugar solutions (25 µl) were pre-incubated for 15 min at 27 °C with 25 µl of the lectin (8 U). Erythrocyte suspension (50µl) was then added, mixed and the plates read after one hour. The glycoproteins were desialated by incubating in 0.1 M H_2SO_4 at 60 °C for 1 h followed by dialysis against distilled water.

Protein determination

Protein concentrations were determined according to Bradford (9) using BSA as standard.

Microorganism and growth

The *Fusarium* sp. (LR11/ NCIM 1330) was routinely maintained on PDA slants (potato 20% w/v, dextrose 1% w/v and agar 1.5% w/v). The fermentation was carried out by inoculating a small piece of mycelium from a 7-day old PDA slant, into 100 ml liquid MGYP medium (malt extract 0.3% w/v, yeast extract 0.3% w/v, peptone 0.5% w/v and glucose 1% w/v), in 500 ml Erlenmeyer flasks, followed by incubation under stationary condition at 26±1 ºC for 5 days. After the fermentation period, the mycelium was collected by filtration, washed three times with glass distilled water, and used as the source of lectin.

Extraction and purification of lectin

All purification steps were carried out at 10±1 ºC. During purification the lectin activity was monitored by hemagglutination assay.

The mycelia (10 gm wet weight) was suspended in 100 ml of 20 mM Tris-HCl buffer, pH 8.0 containing 500 mM NaCl and homogenized. The homogenate was stirred for 5-6 h and then centrifuged (10,000 *g*, 15 min). The pellet was then reextracted as described above. The supernatants were pooled and used for subsequent steps.

The crude extract, obtained from the above step, was dialyzed extensively against 20 mM Tris-HCl buffer, pH 8.0, containing 1.4 M ammonium sulfate and loaded on a Phenyl-Sepharose column (1.5×9 cm) pre-equilibrated with the same buffer at a flow rate of 10 ml/h. The column was then washed with the same buffer till A_{280} of the fractions was < 0.05. The bound lectin was eluted by double gradient *i.e.* decreasing concentration of ammonium sulfate (1.4 M → 0 M) and increasing concentration of ethylene glycol (0% → 10%) at a flow rate of 10 ml/h. Fractions of 2 ml were collected and checked for hemagglutinating activity and protein. Fractions showing hemagglutinating activity were pooled and dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 1.4 M ammonium sulfate and rechromatographed on the same Phenyl-Sepharose column as described above. The bound lectin was eluted by a decreasing gradient of ammonium sulfate (1.4 M → 0 M) at the flow rate of 10 ml/h. Fractions of 2 ml were collected and those showing hemmaglutinating activity were pooled, concentrated by ultrafiltration and loaded on a Sephacryl S-200 column (1.75×108 cm) pre-equilibrated with 20 mM Tris-HCl buffer, pH 8.0 containing 150 mM NaCl and eluted with the same buffer at the flow rate of 10 ml/h. Fractions (1 ml) were collected and those showing hemagglutinating activity were pooled and stored at −20 °C till further use.

Electrophoresis

Native PAGE of the purified lectin was carried out in 8% (w/v) polyacrylamide gel, pH 4.3, according to Reisfeld *et al.* (10) and the gels were stained with Coomassie Brilliant Blue R-250. SDS-PAGE was performed in 15% (w/v) polyacrylamide gel at pH 7.2 according to Weber and Osborn (11). After electrophoresis the gels were visualized by silver staining according to Blum *et al.* (12). Isoelectric focusing (IEF) in polyacrylamide gels was done according to Vesterberg (13) over the pH range 3-10.

Carbohydrate content

Purified lectin (400µg in 400 µl water) was incubated with 400 µl of 5 % (w/v) phenol for 10 min at room temperature. Two ml of sulphuric acid was then added and the mixture was allowed to cool for 20 min at room temperature. The color developed was then measured spectrophotometrically, at 490 nm by using galactose-mannose (4:3) as standard (14).

Molecular mass determination

Gel filtration : The molecular mass of the purified lectin was determined by HPLC (Waters Corporation) on a gel permeation column (Protein-PAK SW300, 300x7.8 mm) at pH 6.0 and 27 °C using β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa) as reference proteins.

SDS-PAGE : This was carried out in 15 % (w/v) polyacrylamide gels, at pH 7.2, according to Weber and Osborn (11) using glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa).

MALDI-ToF : Molecular mass of the purified lectin was determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry using a Voyager DE-STR (Applied Biosystems) equipped with a 337-nm nitrogen laser.

Amino acid analysis

The amino acid analysis was done using amino acid analysis kit AccQ-Fluor supplied by Waters Corporation. Salt free lyophilized lectin (50 µg) was hydrolysed using 6 N constant boiling HCl, in vacuum sealed hydrolysing tubes for 24 h at 110 °C. The sample was then derivatized by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in borate buffer at pH 9 and 10 picomoles of the hydrolysate were loaded on AccQ-Tag column equipped with a fluorescent detector. Total cysteine was determined

according to Cavallini *et al.* (15) and total tryptophan according to Spande and Witkop (16).

N-terminal sequence analysis

N-terminal sequence was determined by automatic Edman degradation by use of a Procise^{TM} protein sequencer (Applied Biosystem) at the protein sequencing facility of IIT, Mumbai, India. The sample (700 pmol) was applied to a glass-fiber filter and then cycled through a conditioning process of fifteen repetitions of Edman chemistry.

Effect of metal ions

The activity of the purified lectin was determined in the presence of 10 mM each of Ca^{++}, Mn^{++}, Mg^{++} and also after dialysis against 10 mM of EDTA.

Temperature and pH stability

Effect of temperature on lectin stability was monitored in the range of 10 °C to 80 °C by incubating 25 µg of the lectin for 15 minutes at the respective temperature, rapidly cooling in ice and assaying for hemagglutinating activity.

The pH stability of the lectin was determined by incubating 25 µg of lectin at different pH (pH 2-12) for 24 h. Buffers used – glycine-HCl (pH 2-3), acetate (pH 4-5), citrate-phosphate (pH 5), phosphate (6-7), Tris-HCl (pH 8-9) and glycine-NaOH (pH 10-12). The hemagglutinating activity was checked after 6, 12 and 24 h.

Preparation of plant polysaccharides (gums)

A number of plant gums were tested for hemagglutination inhibition of the lectin. The suspension of plant gums, gum arabic (0.5 g), gum tragacanth (0.5 g), gum guar (05 g), gum ghatti (0.5 g), gum karaya (0.5 g), gum locust (0.5 g), gum arabinogalactan (0.5 g), gum rosin (0.5 g) and gum pontianac (0.5 g) in 10 ml of distilled water and gum mastic (0.5 g), gum elemi (0.5 g) and gum storax (0.5 g) in 10 ml of 0.1 M NaOH were boiled for 2 h. The suspensions were centrifuged at 7,800 *g* for 30 min at 25 °C and the

supernatants were subjected to alcohol precipitation (1:2 v/v). The precipitate formed was re-dissolved in 10 ml of hot water and reprecipitated with alcohol (1:2 v/v). The precipitate obtained was dissolved in distilled water and dialysed against the same. The concentration of sugar solution were determined in terms of neutral sugar by phenol sulphuric acid method (14).

Chemical modification studies

During chemical modification studies, the residual activity of the modified lectin was determined by hemagglutination assay.

Reaction with PMSF

The lectin (100 µg) in 50 mM Tris-HCl buffer, pH 8.0 was incubated with 5 mM PMSF, at 27±1 °C, for 60 min (17). Aliquots were removed at 15 min intervals, the excess reagent removed by dialysis and residual activity determined. Lectin sample incubated in the absence of PMSF served as the control.

Reaction with DTNB

The lectin (100 µg) in 50 mM phosphate buffer, pH 8.0, was incubated with 0.1 mM DTNB at 27±1 °C for 1 h. Aliquots were removed at different time intervals and the residual activity determined. The modification reaction was also followed by monitoring the increase in absorbance at 412 nm and the number of sulphahydryl groups modified were calculated using a molar absorption coefficient of 13,600 $M^{-1}cm^{-1}$ (18).

Reaction with phenylglyoxal

Arginine residues were modified with phenylglyoxal by the method of Takahashi (19). The reagent was prepared in methanol. The lectin (300 µg), in 50 mM phosphate buffer, pH 8.0, was treated with varying concentrations of phenylglyoxal (0.5-3.0 mM) for 30 min at 25 °C. Excess reagent was then removed by dialysis, and the residual hemagglutination activity determined.

Lectin sample incubated in the absence of phenylglyoxal served as control. The methanol concentration in the reaction mixture did not exceed 2 % (v/v) and had no effect on the activity and stability of the lectin during the incubation period.

Reaction with WRK

The modification of carboxylate residues with Woodward's reagent-K was carried out by incubating 300 µg of the purified lectin, in 50 mM phosphate buffer pH 7.0, with different concentrations (0.01-0.3 mM) of WRK. Aliquots were removed after every 10 min and the reaction stopped by addition of 100 mM acetate buffer, pH 4.5. The reaction mixture was dialysed to remove excess reagent and the residual activity determined. Lectin samples incubated in the absence of WRK served as control. The number of carboxylate groups modified were determined spectrophotometrically, at 340 nm, by assuming a molar absorption coefficient of 7000 $M^{-1}cm^{-1}$ (20).

Reaction with EDC/NTEE

The lectin solution (200µg), in 1 ml 50 mM MES/HEPES buffer, pH 6.0 was incubated with 50 mM EDC and 30 mM NTEE at 27±1 °C for 45 min. After the incubation period, a 100 µl aliquot was taken and the excess reagent was removed by gel filtration on Sephadex G-25 column and the residual hemmaglutinating activity was determined. The lectin sample in same buffer incubated for the same time and temperature without EDC and NTEE served as control. Subsequently the reaction was arrested by the addition of 10 % (w/v) TCA and the precipitated protein was collected by centrifugation, washed extensively with chilled acetone, air dried and dissolved in 100 mM sodium hydroxide. The number of nitrotyrosyl groups incorporated was determined spectrophotometrically, at 430 nm, using a molar absorption coefficient of 4600 $M^{-1}cm^{-1}$ (21).

Modification of lysine

Estimation of lysine with Trinitrobenzenesulphonic acid. (TNBS) :

The reaction mixture containing 0.25 ml of lectin (50 μg) and 0.25 ml 4% (w/v) sodium bicarbonate was incubated with 25 μl of 0.5 % (w/v) TNBS at 37 °C, in the dark, for 2 h. The reaction was terminated by adding 0.125 ml HCI (1 N), and 0.25 ml of 10 % SDS was added to dissolve the precipitate formed (22). The number of free amino groups was determined, spectrophotometrically by assuming a molar absorption coefficient of 9950 $M^{-1}cm^{-1}$ for trinitrophenylated lysine at 335 nm.

Acetylation : Acetylation was performed as described by Fraenkel-Conrat (23). To 500 μg of *Fusarium* lectin, in 1 ml saturated sodium acetate, pH 3, aliquots of acetic anhydride (0.1-1 mM) were added over the course of one hour, after each addition, an aliquot was removed and assayed for hemagglutination activity. The numbers of amino groups modified at the end of the reaction by acetic anhydride were estimated by determining the number of free amino group as determined earlier.

Succinilation : This was carried out by the method of Habeeb (24). Lectin (500 μg), in 100 mM sodium carbonate buffer pH 8.5, was incubated with varying concentration of succinic anhydride (0.5-3.0 mM) prepared in dioxane. The residual activity and the number of amino groups modified were determined as described above.

Reductive methylation: This was carried out as described by Means and Feeney (25). To 1 ml of the lectin (0.3 mg/ml) in 200 mM borate buffer pH 9.0, at 0 °C, 0.1 ml of sodium borohydride solution (0.5 mg/ml) was added, followed by 6 aliquots (5 μl each) of 0.35 % (v/v) formaldehyde at 10 min interval. The procedure was repeated using 3.5 (v/v) % formaldehyde. At the end of the reaction, the residual hemagglutinating activity and the number of amino groups modified were determined as

described above. Lectin incubated in the absence of formaldehyde served as control.

Reaction with NBS

The lectin (300 μg), in 100 mM sodium acetate buffer pH 5.0, was titrated with a total of 0.02 mM NBS, prepared in the same buffer. The reagent was added in five installments and the reaction was monitored spectrophotometrically by monitoring the decrease in absorbance at 280 nm. The number of tryptophan residues modified were determined by assuming a molar absorption coefficient of 5500 $M^{-1}cm^{-1}$ (16). The residual activity was determined by hemagglutination.

Reaction with NAI

This was performed as described by Riordan *et al.* (26). The lectin (300 μg) in 50 mM phosphate buffer pH 7.5 was incubated with different concentrations of NAI (0.1-10 mM) at 27±1 °C for 60 min followed by estimation of the residual activity. The excess reagent was removed by gel filtration on Sephadex G-25 column (1×10 cm) pre-equilibrated in the 50 mM phosphate buffer, pH 7.5. The lectin incubated in the absence of NAI served as control. The tyrosine residues modified were determined spectrophotometrically, using a molar absorption coefficient of 1160 $M^{-1}cm^{-1}$ at 278 nm.

Reactivation with hydroxilamine hydrochloride

Excess NAI was removed by dialysis against 20 mM phosphate buffer pH 7.5, from the reaction mixture prior to reactivation. The reactivation of the lectin was carried out by incubating with hydroxilamine (150 mM) in 50 mM phosphate buffer, pH 7.5 for 6 h (27).

Ligand protection

This was carried out by pre-incubating the lectin with Galβ1→3GalNAc (400 fold molar excess) followed by treatment with NAI. Both modified and

unmodified protein samples were dialysed, the residual activity estimated and the number of residues modified determined.

CD measurements

CD spectra of the native and chemically modified lectin samples were recorded on a JASCO-715 spectropolarimeter, at 25 ^{o}C, in the range of 178-260 nm at scan speed of 200 nm/min with a response time of 1 s and slit width 1 nm. A cylindrical quartz cell of 1 mm path length was used. All measurements were made at a lectin concentration of 0.4 mg/ml. For each spectrum, fifteen successive scans were collected and the averaged spectra were used for further analysis. Measurements were made in 50 mM phosphate buffer pH 7.5 and buffer scans recorded under the same conditions were subtracted form the protein spectra before further analysis.

RESULTS AND DISCUSSION

Lectin characterization

The results of a typical procedure for the purification of *Fusarium* sp. lectin is given in Table 2.1.

Table 2.1 : Purification of *Fusarium* lectin

Purification step	Total activity[a] (U)	Total Protein (mg)	Specific activity[b] (U/mg)	Fold Purification	Recovery (%)
Crude extract	2.1×10^5	3.2	7.0×10^4	1	100
Phenyl Sepharose chromatography I	9.5×10^4	1.11	8.5×10^4	1.22	44
Phenyl Sepharose chromatography II	5.8×10^4	0.28	2.1×10^5	3	27
Gel filtration	5.6×10^4	0.25	2.3×10^5	3.32	26

[a] The reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination was expressed as a unit of hemagglutinating activity.

[b] The specific activity of the lectin is defined as units of the hemagglutinating activity per milligram of lectin.

Fusarium lectin was purified approximately 3-fold with an overall recovery of 26 %. The purified lectin moved as a single band in native PAGE indicating its homogeneity (Fig. 2.1A). The molecular mass of the lectin determined by gel filtration was 26 kDa (Fig. 2.2). The molecular mass determined by SDS-PAGE, in the absence and presence of β-mercaptoethanol, showed a single band corresponding to 12.8 kDa (Fig. 2.3A) and it also corresponded to a MALDI-ToF peak at *m/z* 12.7 kDa (Fig. 2.3B). The results of SDS-PAGE, gel filtration, and MALDI-ToF mass analysis indicated that this lectin is a homodimer of 13 kDa subunits with no disulphide linkages. The lectin showed resemblance in the molecular mass with other lectins reported from same group of fungi: Deuteromycetes. Lectins isolated from *Sclerotina minor, S. trifoliorum, S. miyabeana, Botrytis cinerea* are

homodimers of 17 kDa subunits (28). Lectin of *Rhizoctonia solani* is a dimer of 13 kDa subunits, whereas lectin from *Rhizoctonia crocorum* is a tetramer of 11 kDa subunit (29).

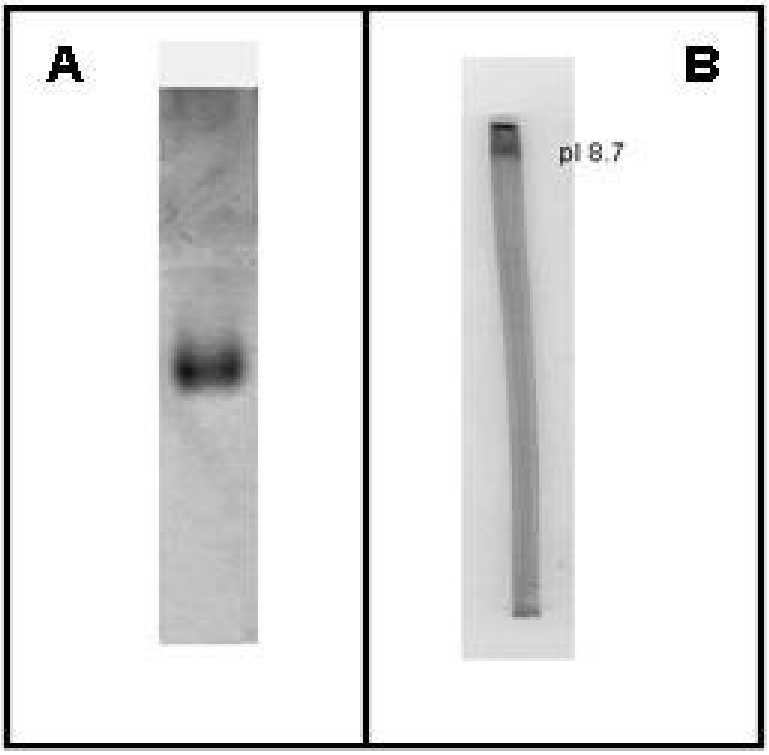

Fig. 2.1. Electrophoresis of purified lectin.

(A) Native electrophoresis of *Fusarium* lectin at pH 4.3.

(B) IEF-PAGE of *Fusarium* lectin. A 50 μg of purified lectin was loaded and carrier ampholines of the range 3-10 were used.

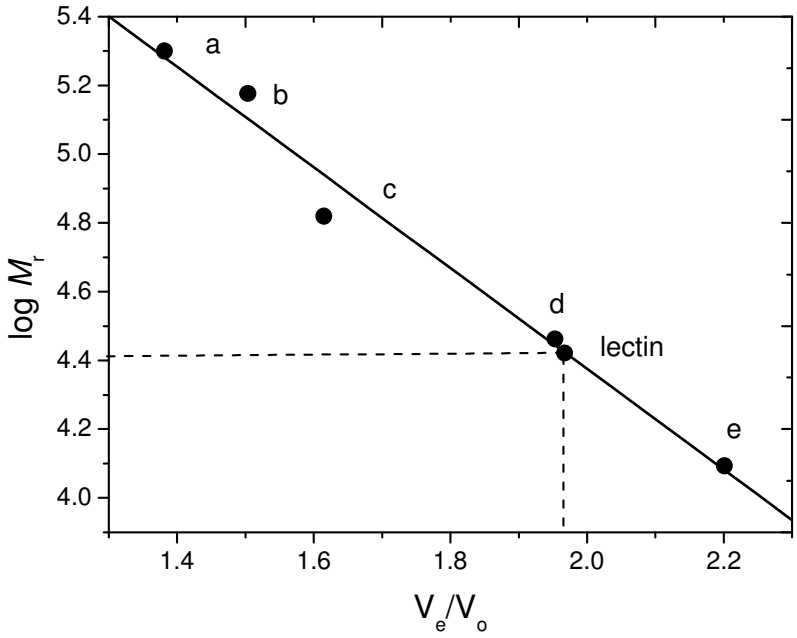

Fig. 2.2 : Molecular mass determination of *Fusarium* lectin by gel-filtration.

(a) β-amylase (200 kDa), (b) alcohol dehydrogenase (150 kDa), (c) bovine serum albumin (66 kDa), (d) carbonic anhydrase (29 kDa) and (e) cytochrome-C (12.4 kDa).

Fusarium sp. lectin was a glycoprotein and contained 3.9% neutral sugar. The amino acid composition of *Fusarium* lectin showed that it contained high amount of tyrosine, lysine and phenylalanine; and two residues of tryptophan and cysteine per dimer (Table 2.2). It was a basic protein with a pI of 8.7 (Fig. 2.1B). Partial N-terminal sequence (first fifteen residues) of the purified lectin was VDVRQIVNLDGLNIV and did not show similarity to any known lectin.

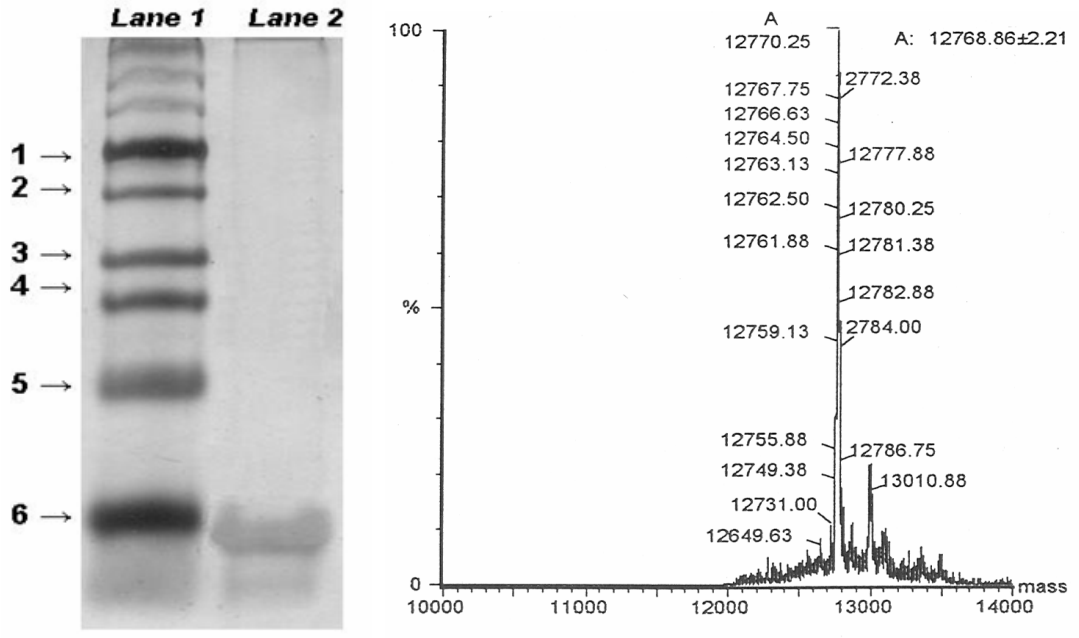

Fig. 2.3 : Subunit molecular mass determination of the lectin

A. SDS-PAGE : Lane 1 : (1) glutamic dehydrogenase (55 kDa), (2) ovalbumin (45 kDa), (3) glyceraldehyde 3-phosphate dehydrogenase (36 kDa), (4) carbonic anhydrase (29 kDa), (5) trypsin inhibitor (20 kDa), (6) α-lactalbumin (14.2 kDa). Lane 2: purified *Fusarium* lectin.

B. MALDI-ToF : Lectin (0.5 µg in 0.5 µl) was applied to a polished stainless steel target and a solution (0.5 µl) of sinapic acid (10 mg/ml) in 30% (v/v) acetonitrile containing 0.1 % TFA, was added. The mixture was allowed to air-dry at ambient temperature. Spectra were acquired in the range of 10 kDa to 100 kDa, on linear mode with delayed ion extraction and with accelerating voltage of 25kV. Low mass ion gate was set to 4500 Da.

Table 2.2 : Amino acid composition of the *Fusarium* sp. lectin

Amino acid	No. of residues/mol
Aspartic acid and asparagine	20
Threonine	24
Serine	14
Glutamic acid and glutamine	16
Proline	10
Glycine	42
Alanine	14
Valine	10
Methionine	6
Isoleucine	8
Leucine	14
Tyrosine	18
Phenylalanine	10
Lysine	30
Histidine	8
Arginine	14
Tryptophan	2[a]
Cysteine	2[b]
Total	**262**

Determined spectrophotometrically by : a. Spande and Witkop (16) b. Cavallini *et al.* (15).

Fusarium sp. lectin showed high pH and thermal stability. It was active between pH 2-12, and temperature upto 70 °C. It lost 25% activity at 80 °C after 15 min; and 90% activity after 30 min. Moreover, it did not show the requirement of divalent cations for its activity since extensive dialysis of the lectin against 10 mM EDTA in 20 mM Tris-HCL buffer, pH 8.0 containing 150 mM NaCl followed by dialysis against plain buffer (to remove EDTA) did not affect the hemagglutinating activity. The far-UV CD spectra was analyzed to derive more quantitative information regarding secondary structural elements of the lectin by using three different methods, viz. CDSSTR (30,31),

CONTINLL (32,33) and SELCON3 (34,35) available at http://lamar.colostate.edu/~sreeram/cdpro/main.html. A basis set containing 43 proteins was used as reference for fitting the experimental spectrum. The results obtained from this analysis are given in Table 2.3. CDSSTR yielded the best fit values and indicated that the *Fusarium* lectin is a predominantly β-sheet protein with a relatively small α-helical content. Moreover, CLUSTER program (36) used for determination of tertiary class of protein showed that lectin belongs to 'All-Beta' class.

Table 2.3 : Results of CD spectral analysis

Method	α_R	α_D	Total α	β_R	β_D	Total β	Turn	Unordered	NRMSD
CDSSTR	0.07	0.09	0.16	0.17	0.09	0.26	0.22	0.36	0.176
SELCON3	0.057	0.078	0.135	0.236	0.117	0.353	0.216	0.216	0.626
CONTINLL	0.080	0.024	0.104	0.208	0.130	0.338	0.336	0.220	0.277
Average			0.133			0.317	0.257	0.265	

α_R and α_D corresponds to regular and distorted α helical structures; β_R and β_D corresponds to regular and distorted β-sheet structures. NRMSD, normalized root mean square deviation.

The lectin did not agglutinate normal or trypsinized erythrocytes but did neuraminidase or pronase-E treated erythrocytes (Table 2.4). The specific activity was approximately 250 times higher with pronase treated erythrocytes than neuraminidase treated erythrocytes. The affinity of lectin for desialated glycoconjugates was also reflected in its better inhibition with desialated glycoproteins. Pronase-E, a cocktail of different proteolytic enzymes, removes all protruding polypeptide from the erythrocyte membrane exposing GPI-anchors and glycolipids, which may serve as better ligands for *Fusarium* lectin than asialo-glycoconjugates on the erythrocyte membrane.

Table 2.4 : Specific activity of *Fusarium* sp. lectin with different blood group erythrocytes treated with different enzymes

Enzymes	Hemagglutinating activity with different erythrocytes (U/mg)		
	A	B	O
Untreated	0	0	0
Neuraminidase	1.16×10^5	1.16×10^5	2.32×10^5
Pronase	3×10^7	3×10^7	3×10^7
Trypsin	0	0	0

For details refer to Methods.

Hemagglutination inhibition

Simple sugars like glucose, mannose, lactose, galactose, rhamnose, xylose, fucose, raffinose, glucosamine, mannosamine, galactosamine, N-acetyl-mannosamine, N-acetyl-galactosamine, N-acetyl-glucosamine failed to inhibit the hemagglutinating activity of the *Fusarium* lectin whereas glycoproteins *viz*. fetuin, asialofetuin, fibrinogen, asialo-fibrinogen, thyroglobulin, holotransferrin, BSM and asialo-BSM; and plant polysaccharide like, gum karaya and gum ghatti were inhibitory. Among the glycoproteins asialofetuin and asialo-BSM were the best inhibitors with minimum inhibitory concentration of approximately 0.1 μg. It was noted that the minimum inhibitory concentration of desialated glycoproteins was 150-300 times lower than the sialated one (Table 2.5).

Glycoproteins possess different N-linked and O-linked glycans, as ligands for lectins to interact. Asialofetuin has three triantennary N-linked core structure with a terminal Galβ1→4GlcNAc and three O-linked structure Galβ1→3GalNAcα1→*O*Ser/Thr (T_α Antigen), while the asialofibrinogen has a biantennary N-linked structure with terminal Galβ1→4GlcNAc residues (37). On the other hand, the Asialo-BSM has several

Galβ1→3GalNAcα1→*O*Ser/Thr and GalNAcα1→*O*Ser/Thr (Tn antigen) structures (38,39). In the present case, the inhibition data suggested that the lectin might be recognizing the complex N-linked as well as O-linked structure.

Table 2.5 : Inhibition of hemagglutination activity of *Fusarium* sp. lectin with different glycoproteins and polysaccharides

Inhibitor	Minimum inhibitory amount* (μg)
Fetuin	15.6
Asialofetuin	0.1
Fibrinogen	30.2
Asialofibrinogen	0.4
Thyroglobulin	0.9
Holotransferrin	62.5
BSM	5.0
Asialo-BSM	0.15
Gum karaya[$]	3.06
Gum ghatti[$]	7.6

Simple sugars, gum elemi, gum arabinogalactan, gum rosin, gum mastic, gum arabic, gum tragacanth, gum locust bean and gum xanthan were not inhibitory.

* Amount of inhibitor that can cause inhibition of 8 U hemagglutinating activity of neuraminidase treated type O human erythrocytes.

[$] In terms of neutral sugar content.

The fungal lectin isolated from *Sclerotium rolfsii* showed similar observations, its activity was inhibited by glycoproteins containing T_α and Tn

structures as asialo-PSM, asialo-BSM and asialofetuin, whereas the sialated glycoproteins showed low inhibitory effect (40). The lectin from *Beauveria bassiana* was found to interact with glycoproteins containing only O-linked structures but not N-linked (41). Contrary, the lectin from opportunistic fungus *Aspergillus fumigatus* (42) showed inhibition with sialated glycoproteins whereas hemagglutinating activity of *Rhizoctonia solani* (43) lectin was inhibited only by simple sugars as N-acetylgalactosamine, galactose, mellibiose, raffinose and others, but not by glycoproteins as fetuin, asialofetuin, ovomucoid and thyroglobulin.

The lectin activity was inhibited by plant polysaccharides such as gum ghatti and gum karaya. Gum ghatti has a backbone chain of (1-6)-linked β-D-galactopyranosyl units with some (1-4)-D-glucopyranosyluronic acid units, some joining (1-2)-D-mannopyranosyl units, and some L-arabinofuranose units. Gum karaya consists of D-galactose, D-glucoronic acid and L-rhamnose but the details of molecular structures are still not known completely (44).

Protein carbohydrate interactions between microbial lectin and plant cell wall polysaccharides have been put forward as the basis of the host pathogen recognition process. The lectin produced by *Xanthomonas campestris* is inhibited by host tissue extract (45); similarly tobacco tissue extract inhibited the Lectin I activity of *Agrobacterium radiobacter* (46). A bacterial lectin from *Agrobacterium tumefacience* (47), specific for L (–) fucose, has been shown to be inhibited by poplar plant (specific host) polysaccharides, and the lectin from phytopathogen *Aspergillus niger* (48) is also inhibited by plant polysaccharides, thus substantiating the above contention. However, several other types of molecules have also been found responsible during early event of infection. It has also been suggested, in case of bacterial infection, that sugars like glucose, galactose and arabinose, or amino acids like valine and arginine may act as chemoattractants (49). Moreover, polysaccharides associated with lectins have also been found to be involved in host-pathogen interactions. For example, the fungal plant pathogen– *Sclerotium rolfsii* produces a lectin with molecular mass of 45 kDa, that is specific for mucin and it is strongly associated with the

polysaccharide (1,3-β-glucan) it produces. The lectin as well as the polysaccharide have a role in pathogenesis (50). It was observed by Gould and Northcote that an integrated fungal molecule containing both carbohydrate and protein, was responsible for adhesion of *Phialophora radicicola* to the host (51).

Lectins have also been found in symbiotic association of microorganisms and host plant. In symbiotic association of *Rhizobium*, a plant lectin has been shown to be a host range determinant (52); but the symbiotic relationship between *Bradyrhizobium japonicum* and soybean plant has been shown to involve a lectin-carbohydrate interaction in which the lectin is produced by the microorganism (53).

Chemical modification

Arginine, cysteine, histidine, lysine, aspartate, glutamate and tryptophan were not found to be involved in hemagglutinating activity of the lectin (Table 2.6). Purified lectin when incubated with 10 mM NAI lost 90% of its initial activity and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in the control samples. Based on a molar absorption coefficient of 1160 $M^{-1}cm^{-1}$ for tyrosine at 278 nm (26) and the molecular mass of 26 kDa for *Fusarium* lectin, the total number of tyrosine residues modified were found to be 8.0. However, the plot of percent residual activity against the number of tyrosine residues modified revealed that the loss of hemagglutination activity occurred due to the modification of 2.3 residues suggesting probable involvement of 2 residues/mol in the hemagglutination activity of *Fusarium* sp. lectin (Fig. 2.4).

Table 2.6 : Effect of different modifying reagents on the activity of the lectin.

Modification reaction	No of residues modified per molecule	Residual activity (%)
Control	0	100
Serine (PMSF)	ND	100
Cysteine (DTNB)	2	100
Arginine (Phenylglyoxal)	ND	100
Tryptophan (NBS)	2	100
Carboxylate (EDAC + NTEE)	4	100
Lysine (Acetic anhydride)	16	100
Lysine (Succinic anhydride)	14	100
Lysine (Methylation)	16	100
Histidine (DEP)	ND	100
Tyrosine (NAI)	8	10
Ligand protection (Lectin+ Galβ1→3GalNAc + NAI)	5.8	25
Reactivation (NAI modified lectin + Hydroxilamine)	--	100

ND- Not determined

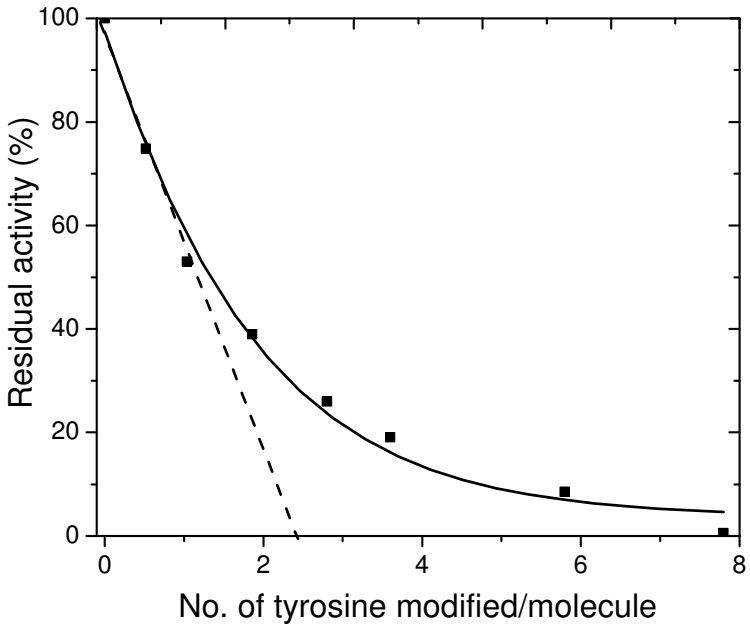

Fig 2.4 : Plot of percent residual activity versus number of tyrosine residues modified : The number of tyrosine residues modified were estimated as described in Methods.

Treatment of tyrosine modified lectin with 0.15 M hydroxylamine for 6 h (which reverse the modification) led to a complete recovery of the hemagglutinating activity of the lectin, indicating that the phenolic group of the tyrosine is primarily involved in the activity of the lectin, and the loss of the hemagglutinating activity is due to the O-acetylation of Tyr residues. NAI mediated inactivation was reduced by 25 % on incubation of lectin with excess of Galβ1→3GalNAc (400 fold molar excess) prior to modification (Table 2.6). Moreover, the CD spectra of both native and modified lectin were almost identical showing that the loss of activity is due to the modification of tyrosine residues rather than structural changes (Fig. 2.5).

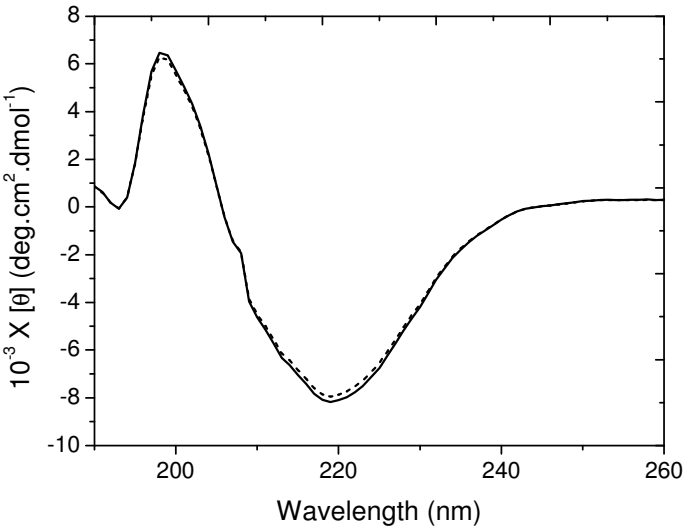

Fig. 2.5 : The CD spectra of native and NAI modified lectin : The CD measurements were performed in a 1mm cell at a lectin concentration 19 μM. Native lectin (—), NAI treated lectin (---).

Tyrosine has been implicated in the sugar-binding activity of a number of lectins as from *Artocarpus hirsuta* (54), *Erythrina indica* (55) and *Trichosanthes dioica* (56).

In conclusion, the present studies showed a lectin is produced by *Fusarium* sp. LR11 having complex sugar specificity. It agglutinates pronase or neuraminidase treated human erythrocytes, and the hemagglutination activity does not require any metal ion. Its hemagglutinating activity can be inhibited by glycoproteins and plant polysaccharides. Chemical modification studies, on *Fusarium* lectin, revealed that tyrosine is present at the sugar binding site of the lectin.

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CHAPTER : 3

CARBOHYDRATE BINDING AND SOLUTE QUENCHING STUDIES OF THE *FUSARIUM* LECTIN BY FLUORESCENCE SPECTROSCOPY

SUMMARY

The intrinsic fluorescence intensity of *Fusarium* lectin was quenched upon binding to disaccharides and glycans, without any change in the emission maximum (348 nm). The quenching observed with asialo-triantennary was 5%. The lectin interacted very poorly with monosaccharides but well with disaccharides indicating an extended binding site. Thermodynamic studies revealed that the binding of the lectin with all the saccharides is enthalpically driven and exothermic in nature. The asialo-triantennary glycan showed 5 fold higher affinity than asialo-biantennary glycan without any significant increase in binding enthalpy, pointing towards the importance of multivalency in the lectin-ligand interactions.

Solute quenching studies of the lectin using acrylamide, succinimide, potassium iodide and cesium chloride were carried out in the absence and presence of asialo-triantennary glycan and at different pH. This studies showed that the single tryptophan residue of the lectin (per monomer) is relatively exposed, and could be in the vicinity of positively charged amino acid residues.

INTRODUCTION

Recognition is a key event in biological functionality of the lectins. In fungi by recognizing different carbohydrate structures present on the surface of their own or host cells, lectins play their roles. Some of these roles seem to concern the fungal metabolism itself, while other activities are implicated in symbiotic or parasitic relationships with other organisms (1). The overall biological activities of the lectins are manifestation of their specificities.

It is essential to understand the mechanism of ligand binding to lectin, in order to facilitate their use as an analytical tool and for better understanding of lectin interaction with cell bound carbohydrates. Determination of association constants with a series of ligands provides considerable insight into the spatial features of a lectin's combining site. Complementary

thermodynamic data offers information on the forces involved in the binding and explains affinity differences encountered.

Since hemagglutination-inhibition studies provide semi-quantitative information, fluorescence spectroscopy has been used to obtain more quantitative information about a lectin's binding process. The advantage of using fluorimetry in studies of carbohydrate-protein interactions is that the binding can be studied at equilibrium without physical separation of the bound complex from the free ligand and the protein (2). Fluorescence of tryptophan is influenced by its microenvironment, hence changes which affect the tryptophan environment can cause changes in fluorescence properties (3).

Fluorescence quenching is a process, which decrease the intensity of the fluorescence emission. The accessibility of tryptophan in a protein molecules can be measured by use of quenchers perturbing fluorescence. Quenching by small molecules either in the solvent or bound to the protein in close proximity to the fluorophore can greatly decrease the quantum yield of a protein. Solute quenching of tryptophan fluorescence provides information on the microenvironment of these residues.

In this chapter, determination of the binding constants for the association of ligand with the *Fusarium* lectin was carried out by ligand-induced quenching of the protein. Thermodynamic parameters for saccharide binding have also been determined and the accessibility of tryptophan residue of the lectin has been studied.

MATERIALS

Galactose, galactosamine, 2-deoxygalactose, L-fucose, glucose, mannose, methyl-α-D-galactose, methyl-β-D-galactose, mellibiose, lactose, pronase-E, carboxypeptidase, aminopeptidase, all glycoproteins, acrylamide, CsCl, KI and succinimide (Sigma Chemical Co. St. Louis, U.S.A); Galβ1→3GalNAc, Galβ1→3GalNAcα1→*O*Ser and Galβ1→4GlcNAc (Dextra Labs, London, UK); Sephadex-G25 (Amersham Bioscience, Uppasala, Sweden); were used. All other reagents were of analytical grade.

METHODS

Protein determination

Protein concentrations were determined according to Bradford (4) using BSA as standard.

Purification of *Fusarium* lectin

Cultivation of *Fusarium* sp. and purification of lectin was carried out as described earlier (Chapter 2).

Neutral sugar estimation

The sugar solutions (400µl) were incubated with 400 µl of 5% (w/v) phenol for 10 min at room temperature. Two ml of sulfuric acid was then added and the mixture was allowed to cool for 20 min at room temperature. The colour developed was then measured spectrophotometrically, at 490 nm, using galactose-mannose (4:3) as standard (5).

Preparation of glycans

One gm of the glycoprotein was dissolved in 100 ml of 20 mM Tris-HCl (containing 150 mM NaCl, 0.5 % w/v sodium azide), pH 7.2 and digested by 50 mg of pronase-E at 37 °C for 72 h, 20 mg of pronase was added after every 24 h. The digest was lyophilized, dissolved in 5 ml of 100 mM acetic acid, centrifuged (10000 ***g***, 20 min), supernatant was collected. The pellet was re-extracted five times in 1 ml of 100 mM acetic acid. Two ml of clear supernatant was loaded on Sephadex G-25 column (1.5 × 100 cm) pre-equilibrated with 20 mM acetic acid, and eluted with the same buffer at the flow rate of 20 ml/h. The fractions (2 ml) were collected and those showing presence of sugar were pooled and further digested by carboxypeptidase (10 U at pH 7.0 and 25 °C for 24 h) and aminopeptidase (10 U at pH 8.5 and 25 °C for 24 h). The residual peptides were removed by chromatography on Dowex-50 column (1.5 × 4 cm) in 20 mm acetic acid (6,7). Desialation of glycopeptides was carried out by incubating with 5 U of neuraminidase in 20

mM Tris-HCl buffer, pH 7.2 at 37 °C for 4 h, the enzyme and sialic acid were removed by successive chromatography on Sephadex G-25 (1.5 × 10 cm) and Dowex-50 as described above.

Fluorescence measurements and ligand-binding data analysis

Fluorescence measurements were carried out using a Perkin Elmer LS-50B spectrofluorimeter, with slit width of 7 nm for both the monochromators and scan speed 100 nm/min. *Fusarium* lectin samples (2 μM) in 10 mM phosphate buffer, pH 6.0 (containing 150 mM NaCl) were placed in a quartz cuvette maintained at desired temperature (± 0.1 °C) by means of a Julabo circulating cryobath. The sugar solution was added in 10-12 aliquots (5 to 10 μl each). Concentration of the stock solutions was in the range of 20-100 mM. Samples were excited at 280 nm and the emission spectra were recorded for wavelength ranging from 300 nm to 400 nm. Each spectrum was an average of 5 accumulations. The fluorescence intensity at 348 nm (λ_{max} of the lectin) was considered for further analysis. Corrections were also made to compensate the dilution effect upon addition of sugar to lectin. At the highest concentration of the saccharide to lectin, volume change was less than 5 % of the solution in the cuvette. Each data point was an average of three independent sets of experiments with SD less than 5%.

The association constants were calculated according to the method described by Chipman *et al.* (8). The abscissa intercept of the plot of log $[C]_f$ against log $\{(\Delta F)/(F_c - F_\infty)\}$, where $[C]_f$ is the free ligand concentration, yielded pK_a value for lectin-ligand interaction according to the relationship (8),

$$\log [F_0 - F_c/F_c - F_\infty] = \log K_a + \log \{ [C]_t - [P]_t (\Delta F/\Delta F_\infty) \} \quad (1)$$

Where F_c is the fluorescence intensity of the lectin at any point during the titration, $[P]_t$ is the total protein concentration, ΔF_∞ is the change in fluorescence intensity at saturation binding, $[C]_t$ is the total ligand concentration, and $[C]_f$ is the free ligand concentration, given by,

$$[C]_f = \{[C]_t - [P]_t (\Delta F/\Delta F_\infty)\} \quad (2)$$

Free energy changes of association (ΔG) were determined by the equation,

$$\Delta G = - \mathrm{RT} \ln K_a \quad (3)$$

Temperature dependence of the association constants was used to determine the thermodynamic parameters. Changes in enthalpy (ΔH) were determined from the Van't Hoff plots by using the equation,

$$\ln K_a = (-\Delta H/\mathrm{RT}) + \Delta S/R \quad (4)$$

Where ΔH is enthalpy change, R is gas constant, ΔS is entropy change and T is the absolute temperature. The entropy change was obtained from the equation,

$$\Delta G = \Delta H - \mathrm{T}\Delta S \quad (5)$$

Solute quenching

Titration of *Fusarium* lectin with acrylamide, CsCl, KI and succinimide were performed in the absence and presence of 100 μM asialo-triantennary glycan. The iodine solution contained sodium thiosulfate (200 μM) to suppress tri-iodate formation. Defined amounts of the quencher (5-10 μl) were added from a stock of 5 M to 2 ml (2μM) lectin solution. The titrations were carried out at pH 4 (acetate buffer), pH 7 (phosphate buffer) and pH 10 (glycine-NaOH buffer), 27°C. The fluorescence spectra were recorded after 3 minutes and each spectrum was an average of 3 accumulations. The excitation wave length was 280 nm and slit width was 7 nm, the emission spectra were recorded in the range of wavelength 300 to 400 nm with slit width of 7 nm and scan speed 100 nm/min. Quenching data for all the quenchers used in this study were analyzed by the Stern-Volmer equation (6) as well as by the modified Stern-Volmer equation (7) (9,10).

$$F_0/F_c = 1 + K_{sv}[\mathrm{Q}] \quad (6)$$

$$F_0/\Delta F = f_a^{-1} + (K_q f_a)^{-1} [\mathrm{Q}]^{-1} \quad (7)$$

Where F_0 and F_c are the respective fluorescence intensities, corrected for dilution, in the absence and presence of quencher, [Q] is the resultant quencher concentration, K_{sv} is the Stern-Volmer quenching constant of the

lectin for given quencher, f_a refers to the fraction of the total fluorescence that is accessible to the quencher and K_q is the corresponding quenching constant. Slopes of Stern-Volmer plots yield K_{sv} values, whereas the slopes of modified Stern-Volmer plots give $(K_q f_a)^{-1}$ and their ordinate give values of f_a.

RESULTS AND DISCUSSION

Ligand Binding

Fluorescence titration of the purified lectins with sugars and glycans resulted in quenching of the lectin fluorescence without any shift in the emission maximum. The maximum quenching of the intrinsic fluorescence of the lectin, on binding with sugar, was 5% (Fig. 3.1). The slope of the plot of log $[F_0-F_c/F_c-F_\infty]$, versus log $[C]_f$ was near unity for all the ligand used, indicating one binding site per monomer of the protein (Fig. 3.1). Association constants obtained for the binding of various sugars at 25 °C are listed in Table 3.1. Decrease in K_a with increasing temperature was observed for all the sugar tested. Van't Hoff plots for different sugars were linear ($r > 0.9$), in the range of temperatures studied (Fig. 3.2). Free energy of the binding (ΔG) was negative at all the temperatures, suggesting the spontaneous nature of the binding. Enthalpy change (ΔH) of binding was negative for all the sugars, indicating exothermic and enthalpically driven nature of the binding (Table 3.2). The *Fusarium* lectin bound very poorly to monosaccharides, as depicted, by K_a determined for several sugars. The lectin did not show any binding with Glc/Man or any of their derivatives. Very low binding was observed with Gal, GalNAc, galactosamine and (L)-fucose (6-Deoxy-Gal). The order of binding was galactosamine>Gal>6-Deoxy-Gal>GalNAc. Substitution at C-2 of Gal had marginal influence on its binding to *Fusarium* lectin. Addition of hydrophilic amino group at C-2 position of Gal resulted in two fold increase in the affinity, while substitution with hydrophobic acetamido group at C-2 position resulted in 4.4 fold decrease in the affinity. The C-6 derivative of Gal, fucose (6-Deoxy-Gal) did not alter the effectiveness of the binding, and showed a comparable K_a value to that of Gal, indicating the non-involvement of hydroxyl group of C-6 in the binding of Gal to the lectin. Addition of methyl group at α or β position as in Met-α-Gal and Met-β-Gal abolished the binding completely.

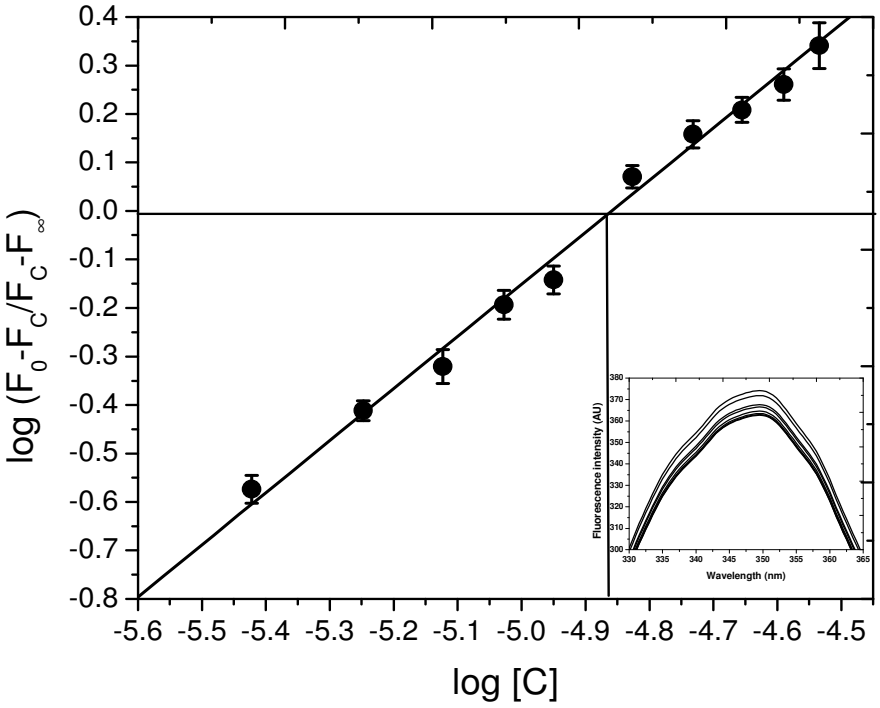

Fig. 3.1 : Determination of the association constant for asialo-triantennary glycan and lectin interaction by fluorescence spectroscopy. F_0 and F_∞ are the fluorescence intensity of free lectin and that bound to the ligand at infinite ligand concentration. F_c is the corrected value of fluorescence intensity at any point of titration, [C] is the ligand concentration in molarity. Inset represents fluorescence quenching of *Fusarium* lectin on addition of aliquots of asialo-triantennary glycan to the lectin solution.

Table 3.1 : Association constants of *Fusarium* lectin with different sugars and glycans determined by fluorescence spectroscopy

Values in parentheses indicate standard deviation (n=3)

	Sugar/Glycan	K_a (M^{-1})	$-\Delta G$ ($kJ \cdot mol^{-1}$)
1.	D-Gal	75	10.69 (±3.5)
2.	D- GalNAc	17	7.02 (±4.5)
3.	Galactosamine	145	12.3 (±2.3)
4.	6-Deoxy L(-) Gal (L-Fucose)	67	10.44 (±3.5)
5.	α Lactose	68	10.45 (±1.9)
6.	Galβ1→3GalNAc	2852	19.71 (±1.5)
7.	Galβ1→3GalNAcα1→*O*Ser	2895	19.74 (±1.8)
8.	Galβ1→4GlcNAc (LacNAc)	1355	17.86 (±2.2)
9.	Asialo-triantennary N- glycan	71610	27.69 (±0.4)
10.	Sialated-triantennary N- glycan	5200	21.23 (±1.2)
11.	Asialo-biantennary N- glycan	14640	23.73 (±0.9)
12.	Sialated-biantennary N- glycan	650	16.04 (±2.1)

Methyl-α-D-Gal, methyl-β-D-Gal, glucose, N-acetylglucosamine, glucosamine, methyl-α-D-Glc, methyl-β-D-Glc, mannose, mannosamine, N-acetylmannosamine, methyl-α-D-Man, methyl-β-D-Man, 6-deoxy-α-(L)-mannose (rhamnose), Galβ1→3GlcNAc (Lacto-N-Biose) and mellibiose did not show considerable change in the fluorescence spectrum of *Fusarium* lectin.

Table 3.2 : Association constants for the binding of glycans to *Fusarium* lectin different temperatures

Glycan	$10^{-3} \times K_a$ (M^{-1})			
	15 °C	20 °C	25 °C	30 °C
Galβ1→3GalNAc	--	3.52	2.85	2.30
Galβ1→3GalNAcα1→*O*Ser	--	3.42	2.89	2.45
Galβ1→4GlcNAc	--	1.58	1.35	1.16
Asialo-triantennary N- glycan	129.3	93.06	71.61	51.1
Sialated-triantennary N- glycan	7.8	6.3	5.2	4.23
Asialo-biantennary N- glycan	--	19.24	14.64	10.62
Sialated-biantennary N- glycan	--	0.66	0.65	0.52

Table 3.3 : Thermodynamic parameters for the binding of glycans to *Fusarium* lectin*

Values in parentheses indicate standard deviation (n=3)

Glycan	$-\Delta H$ ($kJ \cdot mol^{-1}$)	$-\Delta G$* ($kJ \cdot mol^{-1}$)	$-\Delta S$ ($J \cdot mol^{-1} \cdot K^{-1}$)
Galβ1→3GalNAc	27.16 (±1.4)	19.71 (±1.5)	24.99 (±1.3)
Galβ1→3GalNAcα1 →*O*Ser	22.2 (±1.1)	19.74 (±1.8)	8.27 (±1.3)
Galβ1→4GlcNAc	20.89 (± 2.2)	17.86 (±2.2)	10.16 (±2.4)
Asialo-triantennary N-glycan	44.06 (±0.6)	27.69 (±0.4)	54.01 (±0.4)
Sialated-triantennary N- glycan	29.51 (±1.1)	21.23 (±1.2)	27.02 (±1.1)
Asialo-biantennary N-glycan	43.80 (±0.9)	23.73 (±0.9)	67.31 (±0.8)
Sialated-biantennary N- glycan	29.51 (±2.5)	16.04 (±2.1)	45.2 (±2.6)

* Values at 25 °C.

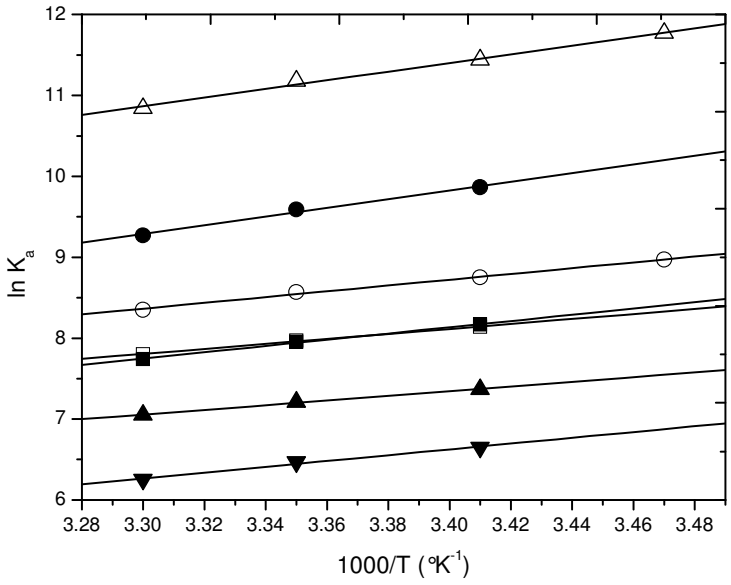

Fig. 3.2 : Van't Hoff plots for the association of various sugars to *Fusarium* lectin are drawn according to the regression equation ($r > 0.9$, $n = 3$). The symbols used are: (■) Galβ1→3GalNAc, (□) Galβ1→3GalNAcα1→*O*Ser, (▲) Galβ1→4GlcNAc, (△) Asialo-triantennary glycan, (○) Sialated-triantennary glycan, (●) Asialo-biantennary glycan, (▼) Sialated-biantennary glycan.

Comparison of association constants for disaccharides Galβ1→3GalNAc (T-antigen) and Galβ1→4GlcNAc (LacNAc) with monosaccharide galactose, showed 38 fold and 18 fold higher affinity, respectively (Table 3.1). Several fold higher affinity for disaccharides suggests that the lectin may also have secondary subsite as observed in the case of plant lectins from *Artocarpus hirsuta* (11), *Maclura pomifera* (12) and Artocarpin (13). Whereas the lectin from fungus *Rhizoctonia solani* (RSA) exhibited specificity for Gal/GalNAc residues, and the affinity for various di- and tri-saccharide was very similar to Gal, suggesting that the carbohydrate-binding site(s) of RSA can accommodate only a single sugar unit (14). On the other

hand, the lectin form phytopathogenic ascomycete *Sclerotinia sclerotiorum* can accommodate disaccharides as they are more potent ligands than Gal/GalNAc (15).

Among the various disaccharides used Galβ1→3GalNAc was the most potent ligand and showed 2 fold higher affinity over Galβ1→4GlcNAc. Although both disaccharides have similar configuration of non-reducing sugar (Gal) but show startling difference in the orientation of the reducing sugar (11,16). This indicate that the lectin can accommodate β1→3GalNAc better than β1→4GlcNAc disaccharides, and the better affinity is also accompanied by enthalpic contribution. Among other di- and oligosaccharides, lactose showed very weak affinity, whereas lacto-N-Biose, melibiose, raffinose, stachyose and trehalose did not show any affinity.

Comparison of binding with Galβ1→3GalNAc (T-antigen) and Galβ1→3GalNAcα1→*O*Ser (T_α antigen) with the *Fusarium* lectin suggested the role of serine in binding (Table 3.1). Addition of Ser to Galβ1→3GalNAc resulted in a slight increase in the affinity constant accompanied by a decrease in enthalpy and increase in entropy. The value of ΔH decreased by a difference of 7 $kJ \cdot mol^{-1}$ with Galβ1→3GalNAcα1→*O*Ser. The entropic contribution of Ser in the binding of Galβ1→3GalNAcα1→*O*Ser is probably based on increased hydrophobic interaction with the amino acids and/or by rearrangement of water molecules near the binding site and/or by loss of structural flexibility of the ligand.

Lectin-carbohydrate interaction are generally characterized by a low affinity for monovalent ligand. In general, millimolar affinity is observed for lectins binding to monosaccharides. When longer oligosaccharides act as ligands, corresponding to an extended binding site on the lectin surface, increased affinity up to micromolar range can be observed Branched complex glycans, due to the clustering effect, offer multiple binding sites leading to several fold increase in the affinity (17).

The N-glycan of fetuin has a triantennary structure with ultimate Galβ1→4GlcNAc, whereas fibrinogen has biantennary structure with ultimate

Galβ1→4GlcNAc (Fig 3.3) (18,19). The O-linked glycan of fetuin, Galβ1→3GalNAcα1→*O*Ser (T_α antigen), is a monovalent structure. The sialated form of the glycan has a terminal sialic acid, as NeuAcα2→3/6Gal (Fig. 3.3).

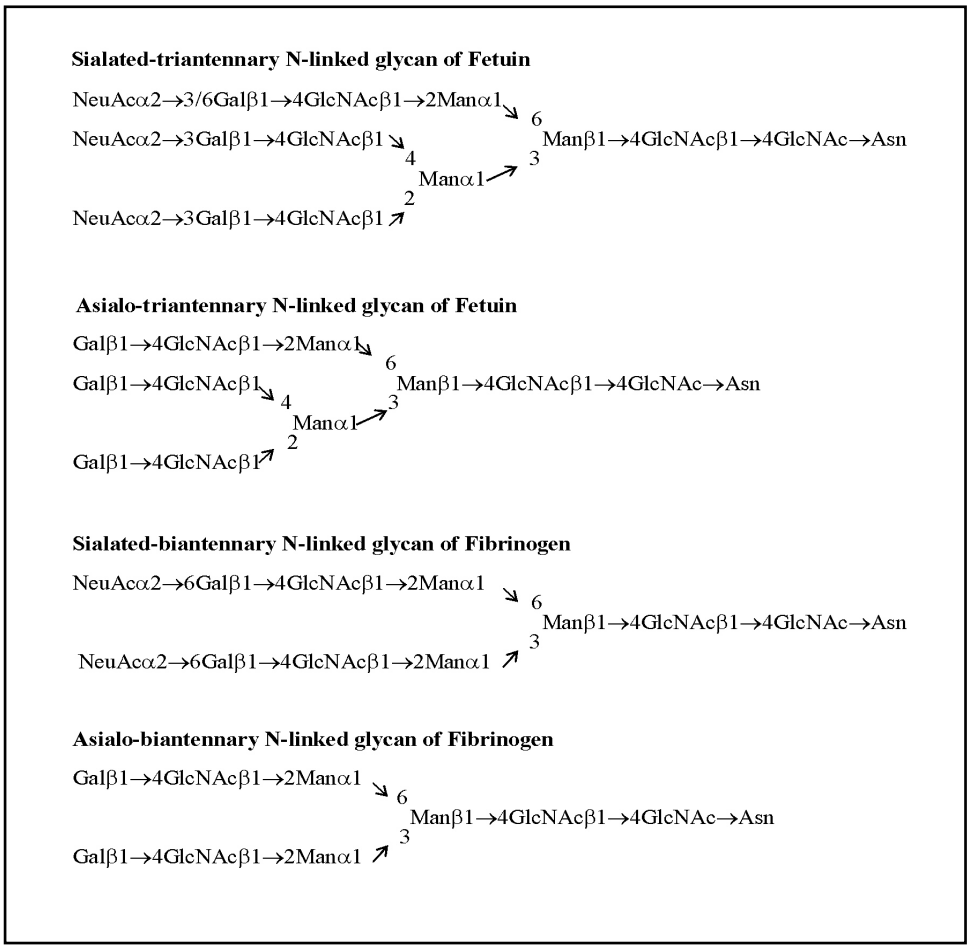

Fig. 3.3 : Structure of different glycans

The glycans were prepared from fetuin and fibrinogen as described in Methods.

The asialo-triantennary glycan exhibited 5 fold higher binding affinity than asialo-biantennary glycan (Table 3.1). However, the higher binding

affinity observed with the former was not accompanied by coincidental enthalpic increase, suggesting no extra hydrogen bond(s) formation on binding. The increase in binding affinity was solely due to increase in the positive entropy of the interaction probably due to decrease in the ligand conformational flexibility in lieu of the increase in branching from biantennary to triantennary.

The higher affinity of the asialo-triantennary and asialo-biantennary glycans is due to increase in enthalpy and negative entropy contribution as compared to monovalent disaccharide Galβ1→4GlcNAc. The favorable energetic contribution responsible for the better affinity of these glycans is probably due to increase in hydrogen bonding with multiantennary glycans. The addition of bulky and negatively charged NeuAc at C-3/6 position in ultimate Gal reduced the binding affinity by 14- and 22- fold with triantennary and biantennary N-glycans respectively. The reduction in the binding affinity is probably due to some unfavorable interaction between NeuAc and lectin or the addition of the bulky NeuAc might be creating some steric hindrance by reducing the accessibility of penultimate Gal to the ligand binding pouch of the lectin. Moreover, probability of unfavorable ionic interaction between the NeuAc and lectin cannot be excluded. Simultaneous reduction of binding enthalpy is also obvious due to loss of contributory hydrogen bonding or van der Walls interaction. Such type of reduction in the affinity with sialated glycoconjugates was also reported from mushroom lectin *Chlorophyllum molybdites* (20), where asialo-fetuin and asialo-BSM (bovine submaxillary mucin) showed 10-15 times higher affinity than their sialated native form. However, higher affinity with sialated glycoconjugates can also be found in lectins, where primary interacting counterpart is NeuAc itself, rather than penultimate Gal residue (21).

Solute Quenching Studies

The tryptophan exposure and its microenvironment in proteins can be investigated by fluorescence quenching, using small molecules as acrylamide, succinimide, iodide, cesium and oxygen termed as quenchers. Titration of the lectin with acrylamide, succinimide, KI and CsCl resulted in 100 %, 57 %, 61 % and 19 % quenching, respectively (Table 3.4). The *Fusarium* lectin was found to have a single, relatively exposed, tryptophan (per monomer), which showed λ_{max} of emission at 348 nm. For a single tryptophan monophasic Stern-Volmer plot can be expected, and indeed it was found in the case of *Fusarium* lectin, as no downward curvature was observed for any of the quenchers studied (Fig 3.4). For acrylamide, there was no considerable difference in the K_q and f_a at different pH and in the presence of the ligand (Fig. 3.5 and Table 3.4). Total accessible fluorophore fraction was near unity. Succinimide, being bulkier, showed 50% quenching of the total available fluorescence. Acrylamide and succinimide both are polar but neutral quenchers so the presence of local charges in the vicinity of tryptophan microenvironment does not affect their quenching ability. The K_{sv} for succinimide (4.75 M^{-1}) as compared to acrylamide (24.8 M^{-1}) at pH 6.0 reflected both the inefficiency of quenching by the former as well as its restricted accessibility to the tryptophan (Table 3.4).

Succinimide has a larger molecular radius than acrylamide, and sterically much more rigid. Hence, its approach to buried tryptophan could be much more hindered and therefore, it is a less efficient quencher than acrylamide (22). Direct Stern-Volmer plot of acrylamide quenching did not show considerable change at different pH and in the presence of ligand, but showed a clear upward curvature, indicating significant contribution from static quenching in the total quenching (Fig. 3.4). The static quenching is shown when a quencher deactivates an excited fluorophore at the instant of excitation, by forming a nonfluorescent or dark complex in the ground state, or by being present within a certain “sphere of action” at the time of excitation (23).

Table 3.4 : Summary of parameters obtained from the intrinsic fluorescence quenching with different quenchers

Quencher and condition	K_{sv} M^{-1}	K_q M^{-1}	f_a
Acrylamide			
pH 4.0	25.5	17.90	1
pH 6.0	24.8	19.43	1
pH 10.0	24.3	18.13	1
With ligand	35.9	20.06	1
Succinimide			
pH 4.0	4.29	14.77	0.49
pH 6.0	4.75	12.06	0.57
pH 10.0	3.50	11.66	0.43
With ligand	4.34	11.75	0.41
KI			
pH 4.0	3.66	6.07	0.67
pH 6.0	2.97	5.73	0.61
pH 10.0	2.08	4.28	0.56
With ligand	2.32	4.74	0.56
CsCl			
pH 4.0	0.60	4.13	0.19
pH 6.0	0.61	4.92	0.19
pH 10.0	0.67	5.63	0.20
With ligand	0.56	4.75	0.19

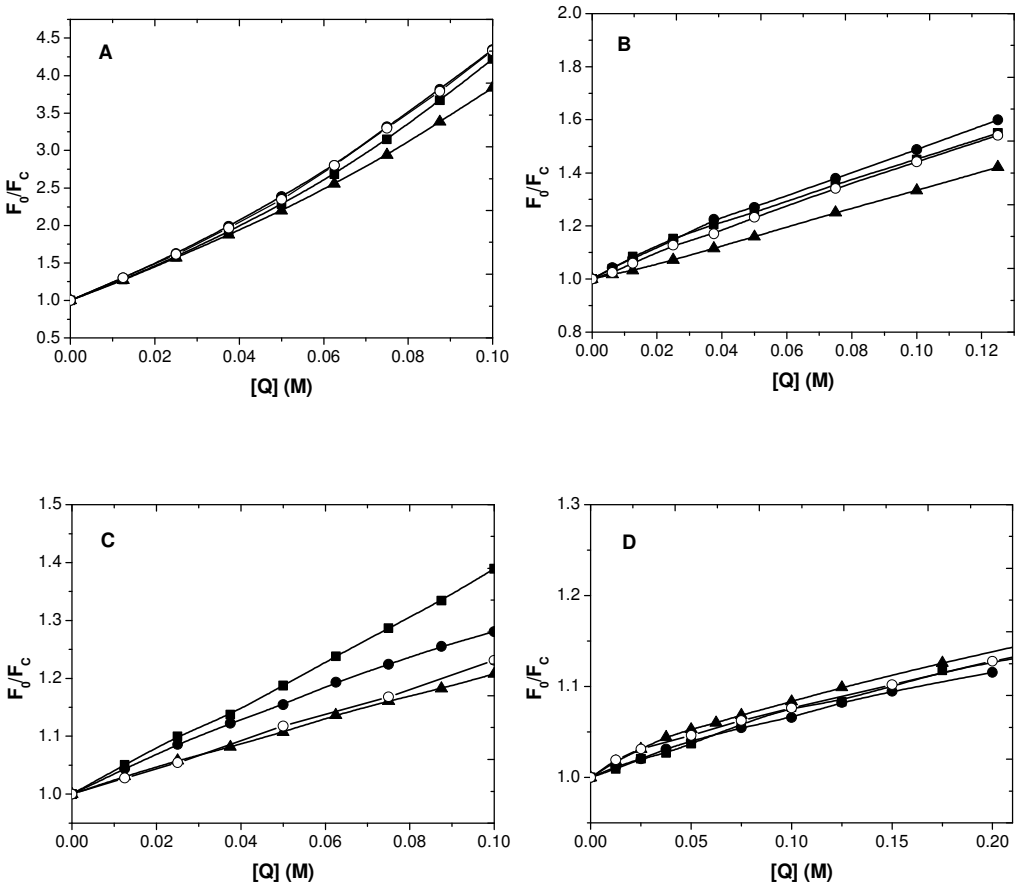

Fig 3.4 : Stern-Volmer plots of fluorescence quenching for *Fusarium* lectin. The different quenchers used are (A) acrylamide (B) succinimide (C) iodide ion and (D) cesium ion. Lectin at (■) pH 4.0, (●) pH 6, (▲) pH 10 and (○) in the presence of ligand.

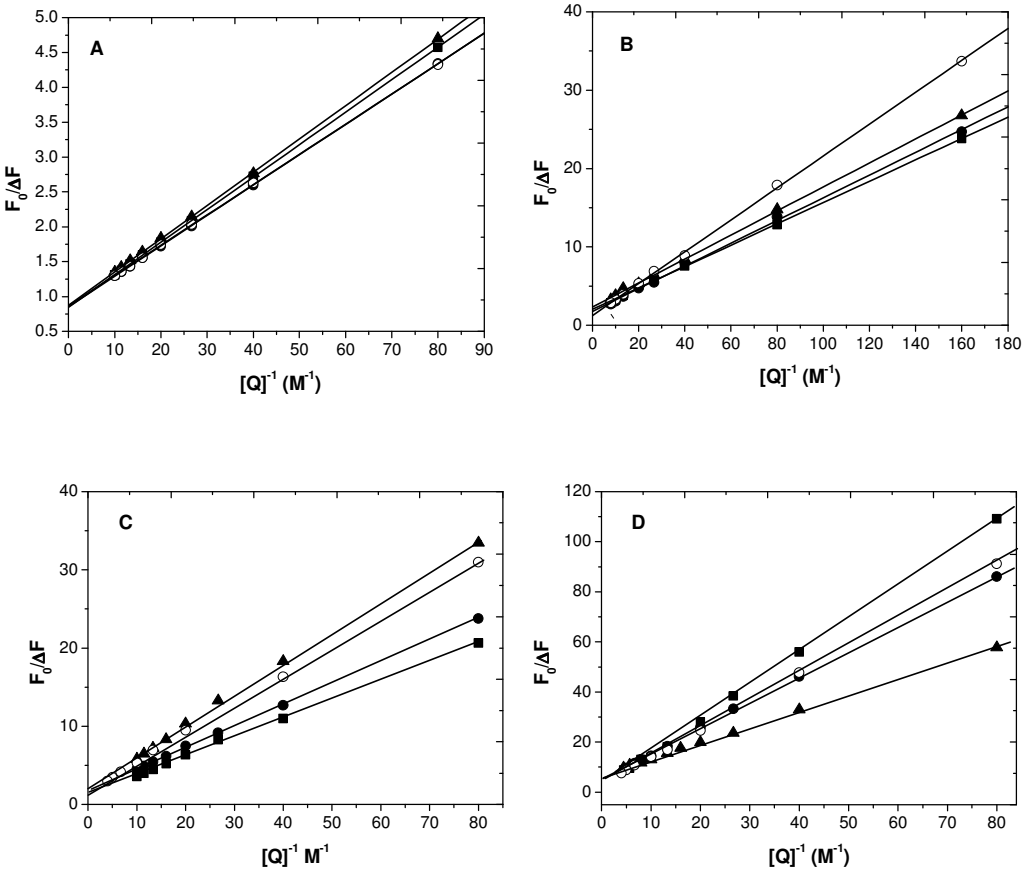

Fig 3.5 : Modified Stern-Volmer plots of fluorescence quenching for *Fusarium* lectin. The different quenchers used are (A) acrylamide (B) succinimide (C) iodide ion and (D) cesium ion. Lectin at (■) pH 4.0, (●) pH 6, (▲) pH 10 and (○) in the presence of ligand.

The ionic quenchers, I^- and Cs^+ being charged, cannot penetrate into the protein interior, unlike the neutral quenchers and thus do not probe buried tryptophan, so they were found less efficient than acrylamide. The ionic quenchers are useful to explore the microenvironment of relatively exposed tryptophan. Their accessibility and quenching efficiency depend on the local charges of tryptophan microenvironment (24,25). Quenching of *Fusarium* lectin with Cs^+ and I^- was carried out at pH 4, 6 and 10. Considerable difference in the quenching and the accessibility was observed with Cs^+ and I^-

at different pH (Fig 3.4 and 3.5). K_{sv} value for I^- (2.97 M^{-1}) and Cs^+ (0.61 M^{-1}), indicates iodide has more accessibility to the fluorophore then cesium (Table 3.4). Cs^+ and I^- exhibited 19 % and 61 % quenching, respectively. In the case of Cs^+ quenching, raising the pH resulted in increased quenching and accessibility (K_{sv} = 0.60 M^{-1} at pH 4 and 0.67 M^{-1} at pH 10). Iodide a negatively charged quencher resulted in opposite effect than Cs^+ as K_{sv} decreased with increase in pH (K_{sv}= 3.66 M^{-1} at pH 4 and to 2.08 M^{-1} at pH 10). Accessibility and quenching efficiency of Cs^+ and I^- with effect of pH suggest that the microenvironment of tryptophan is probably positively charged.

The presence of the ligand in the quenching with succinimide, Cs^+ and I^- resulted in a slight decrease in accessibility, indicating that ligand binding might result in slightly decreased penetration of the quencher into the protein matrix.

In conclusion, the major forces for the binding of different saccharides to *Fusarium* lectin are hydrogen bonding and van der Waals interactions coupled with some nonpolar contribution. The lectin discriminates between $\beta1\rightarrow3$ and $\beta1\rightarrow4$ linked disaccharides. Position of C-4 hydroxyl group and presence of acetamido group, in reducing sugar, are the two main criteria for the difference in the affinity with different disaccharides. The higher affinity accompanied by a large enthalpic contribution observed with complex N-glycans suggested the importance of multivalency in binding. The single tryptophan of the lectin is relatively exposed and accessible to neutral as well as ionic quenchers and it seems to present in vicinity of positively charged amino acids.

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CHAPTER : 4

GLYCAN BINDING TO THE *FUSARIUM* LECTIN: SURFACE PLASMON RESONANCE STUDIES

SUMMARY

The thermodynamics and kinetics of binding of glycans and glycoproteins to *Fusarium* sp. lectin was studied using surface plasmon resonance. The lectin showed very high affinity for asialo-bovine submaxillary mucin (K_a=1.61 × 10^{10} M^{-1} at 25 °C). The affinity for various glycoproteins were in the order of asialo-BSM >> BSM ≅ asialofetuin > fetuin ≅ asialofibrinogen > fibrinogen. Compared to glycans, their corresponding glycoproteins showed several fold higher affinity with significant contribution from enthalpy and positive entropy, suggesting the involvement of non-polar protein-protein interaction. The higher affinity of the glycoproteins was due to their faster association rates and the activation energy in the association process was much lower for the glycoproteins than glycans, resulting in their faster associations. These observations elaborate the role of protein matrix in lectin-glycoconjugate interaction.

INTRODUCTION

In many biological recognition process adhesion involves the formation of saccharide-protein complexes. In order to understand the selectivity and origin of the association energy, it is important to understand the forces controlling saccharide-protein interactions. Surface plasmon resonance (SPR), which permits the real time monitoring of binding, without tagging one of the two components, is convenient tool to acquire such data (1). Moreover, the SPR is also useful in understanding of protein-protein interaction. This Chapter describes studies on the binding of the lectin to different glycans and their corresponding glycoproteins by SPR.

MATERIALS

Fetuin, fibrinogen, mucin, (Sigma Chemical Co. St. Louis, U.S.A); Galβ1→3GalNAcα1→*O*Ser (Dextra Labs, London, UK); Certified grade CM5 sensor chip and amine coupling kit (*N*-ethyl-*N'*-(dimethylaminopropyl-) carbodiimide hydrochloride and *N*-hydroxysuccinamide) (Pharmacia

Biosensor AB, Uppsala, Sweden) were used. All other chemicals used were of analytical grade.

METHODS

Protein determination

Protein concentrations were determined according to Bradford (2) using BSA as standard.

Glycan preparation

Asialo-triantennary glycans from fetuin and asialo-biantennary glycan from fibrinogen were prepared as described in Chapter 3.

Purification of *Fusarium* lectin

Cultivation of *Fusarium* sp. (LR11) and purification of lectin was carried out as described in Chapter 2. The purified lectin was dialysed extensively against deionized water and used for immobilization on the sensor chip.

BIAcore biosensor assays

Biospecific interactions studies were performed on a BIAcore 2000 (Pharmacia Biosensor AB, Uppsala, Sweden) biosensor system based on the principle of surface plasmon resonance. One hundred µg of *Fusarium* lectin in 1 ml of 10 mM sodium acetate buffer, pH 4.0 were coupled (corresponding to 2000 response units), to a certified grade CM5 chip at a flow rate of 5 µl/min for 50 min using the amine coupling kit. The unreacted groups, on the surface of the chip, were blocked with ethanolamine. All measurements were done using 10 mM phosphate buffer, pH 6.0, (containing 100 mM NaCl, 0.02% w/v sodium azide and 0.05% v/v Tween-20). Prior to injection, sugar and protein samples were dissolved and diluted in the above buffer to avoid buffer mismatch. The association rate constants, at different temperatures, were determined by passing the glycan solutions (0.4-600 µM) over the chip at a flow rate of 5 µl/min for 300 s. The dissociation rate constants, on the

other hand, were determined in a similar manner by passing plain buffer at a flow rate of 5 µl/min for 300 s. The glycoproteins were passed at a high flow rate 50µl/min for 120 s to reduce the mass transport effect and dissociation was followed by passing buffer at a flow rate of 50 µl/min for 300 s. After every cycle, chip was regenerated by treating with 200 mM sodium carbonate, pH 9.5, for 2 min.

Data Analysis

Association (k_1) and dissociation (k_{-1}) rate constants were obtained by nonlinear fitting of the primary sensogram data using the BIAevaluation software version 3.1. The dissociation rate constants were derived using the equation,

$$R_t = R_{t0}\, e^{-k_{-1}\ (t - t_0)} \qquad (1)$$

Where R_t is the response at time t and R_{t0} is the amplitude of the initial response. The association rate constant k_1 was derived by equation 2 using the measured k_{-1} values,

$$R_t = R_{\max}\,[1 - e^{-(k_1 C + k_{-1})\,(t - t_0)}] \qquad (2)$$

where $R_{\max}$ is the maximum response and C is the concentration of the analyte (ligand) in the solution. K_a (k_1/k_{-1}) is the association constant. The equilibrium resonance units obtained from the binding interaction of the glycans with the protein on the surface of the chip were also plotted as per Scatchard analysis (3-5) using the equation.

$$R_{eq}/C = K_a\, R_{max} - K_a\, R_{eq} \qquad (3)$$

Where R_{eq} is the equilibrium resonance units, R_{max} is the resonance signal at saturation, C is the concentration of free protein and K_a is the equilibrium association constant.

Free energy changes of association (ΔG) were determined by the equation;

$$\Delta G = -\,RT \ln K_a \qquad (4)$$

Temperature dependence of the association constants was used to determine the thermodynamic parameters. Changes in enthalpy (ΔH) were determined

from Van't Hoff plots by using Equation,

$$\ln K_a = (-\Delta H/RT) + \Delta S/R \quad (5)$$

Where ΔH is enthalpy change, R is gas constant, ΔS is entropy change and T is the absolute temperature. The entropy change was obtained from the equation,

$$\Delta G = \Delta H - T\Delta S \quad (6)$$

Activation enthalpies ($\Delta H^{\ddagger}$), entropies ($\Delta S^{\ddagger}$) and energies were calculated using the following equations:

$$\Delta H^{\ddagger} = E_A - RT \quad (7)$$

$$\ln (k^{\ddagger}/T) = -\Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R + \ln (k'/h) \quad (8)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (9)$$

where $k^{\ddagger}$ is the appropriate rate constants, k' is Boltzman's constant and h is Planck's constant.

RESULTS AND DISCUSSION

Fungal lectins show very high affinity for multivalent ligands *viz*. glycoproteins (6,7). Millimolar affinity is observed for lectins binding to monosaccharides whereas, with longer oligosaccharides, increased affinity (up to micromolar range) can be observed due to an extended binding site (8). These type of interactions, brought about by branched oligosaccharides, are typified by a favorable enthalpy resulting from large number of hydrogen bonds. Such interactions are compensated by an unfavorable entropy contribution, attributed either to solvent rearrangement (9) or to loss of ligand conformation (10).

SPR studies on the interaction of *Fusarium* lectin with different glycans and glycoproteins showed significant changes in RUs (Fig. 4.1 and 4.2). The N-linked, asialo-triantennary and asialo-binantennary glycans showed 25 and 6 fold, respectively higher affinity than O-linked glycan (Table 4.2). The asialo-triantennary glycan exhibited 5 fold higher affinity than asialo-biantennary glycan and the higher affinity was accompanied by a favorable positive entropy rather than any enthalpic increase, suggesting there is no extra hydrogen bond formation with asialo-triantennary glycan. This observation indicates that the increase in affinity is due to an increase in multivalency of the ligand. Moreover, comparable K_a determined by kinetic and Scatchard analysis indicated the monophasic nature of the binding.

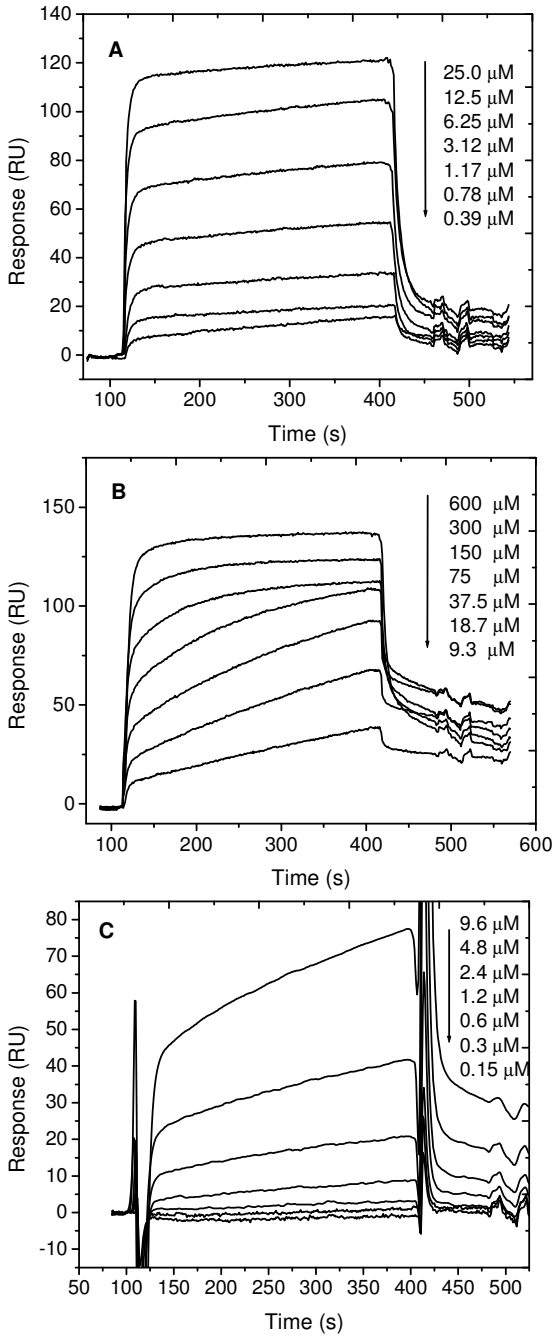

Fig. 4.1 : The Representative sensograms depicting interactions of increasing amounts of glycans to the immobilized *Fusarium* lectin at 25 °C. (A) Asialo-triantennary glycan, (B) Asialo-biantennary glycan, (C) Galβ1→3GalNAcα1→*O*Ser. The glycan solutions were injected for 300 s at a flow rate of 5 μl/min. The dissociation reaction was recorded by flowing the plain buffer at flow rate of 5 μl/min for 300 s. The surface of the chip was regenerated by 10 s pulse of bicarbonate buffer pH 9.5.

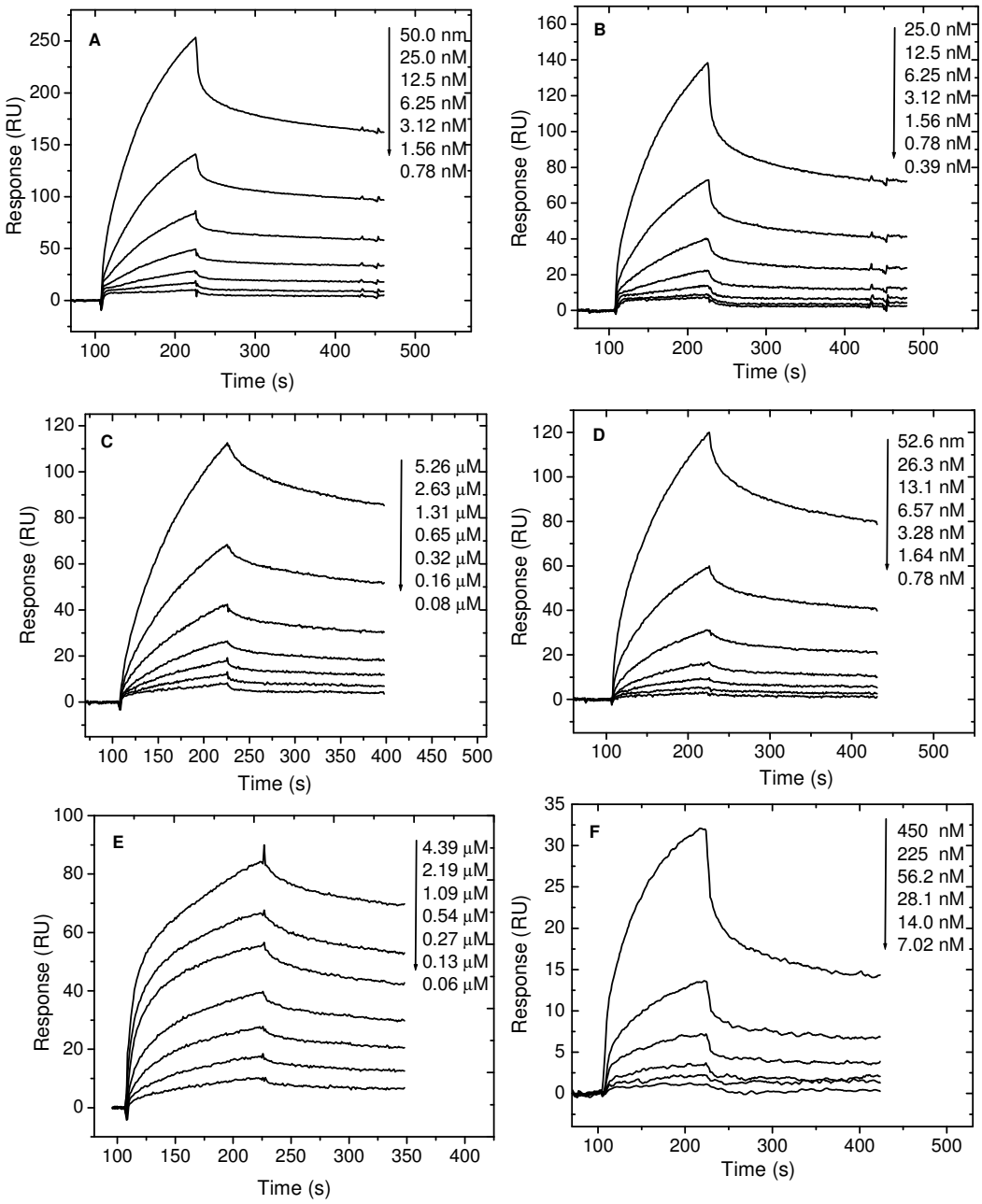

Fig. 4.2 : The Representative sensograms depicting interactions of increasing amounts of glycoproteins to the immobilized *Fusarium* lectin at 25 °C. (A) BSM (B) Asialo-BSM (C) Fetuin (D) Asialo-fetuin (E) Fibrinogen (F) Asialo-fibrinogen. The glycoprotein solutions were injected for 120 s at a flow rate of 50 µl/min. The dissociation reaction was recorded by flowing the plain buffer at flow rate of 50 µl/min for 300 s. The surface of the chip was regenerated by three 10 s pulse of bicarbonate buffer pH 9.5.

Table 4.1 : Association constants for the binding of different glycans and glycoproteins to immobilized *Fusarium* lectin at different temperatures: Determined by SPR (Kinetic analysis)

Ligand	K_a (M^{-1})			
	20 °C	25 °C	30 °C	35 °C
Galβ1→3GalNAcα1 →*O*Ser[$]	3.39×10^3	2.92×10^3	2.49×10^3	--
Asialo-triantennary N-glycan[*]	9.4×10^4	7.37×10^4	5.16×10^4	--
Asialo-biantennary N-glycan [**]	1.92×10^4	1.57×10^4	1.05×10^4	--
Asialo-BSM	--	1.61×10^{10}	1.13×10^{10}	0.62×10^{10}
BSM	--	9.88×10^8	6.27×10^8	4.15×10^8
Asialofetuin	--	8.50×10^8	5.86×10^8	3.69×10^8
Fetuin	--	7.98×10^6	6.12×10^6	4.03×10^6
Asialofibrinogen	--	1.58×10^8	1.13×10^8	0.73×10^8
Fibrinogen	--	2.56×10^6	1.95×10^6	1.34×10^6

$ Data was obtained as per Scatchard analysis

* $K_a = 7.22 \times 10^4$ M^{-1}at 25 °C by Scatchard analysis

** $K_a = 1.46 \times 10^4$ M^{-1}at 25 °C by Scatchard analysis

The lectin showed very high affinity with glycoproteins in the order of asialo-BSM >> BSM ≅ asialofetuin > fetuin ≅ asialofibrinogen > fibrinogen. The binding data with glycoproteins could be obtained only by kinetic analysis and not by Scatchard analysis due to non-saturation of binding. The binding was very high with asialo-BSM (K_a= 1.61×10^{10} M^{-1}) than asialofetuin (K_a=8.50×10^8 M^{-1}) and asialofibrinogen (K_a= 1.58×10^8 M^{-1}) at 25 °C (Table 4.1). The binding of *Fusarium* lectin with sialated glycoproteins as BSM (K_a= 9.88×10^8 M^{-1}), fetuin (K_a= 7.98×10^6 M^{-1}), fibrinogen (K_a= 2.56

$\times 10^6$ M^{-1}), was approximately 20-100 fold less than their desialated counterparts (Table 4.1). The binding enthalpy was negative with all the glycoproteins with a value of ΔH= −64.68, −72.58, −52.06, −64.44, −49.34 and −59.89 $kJ \cdot mol^{-1}$ for BSM, asialo-BSM, fetuin, asialofetuin, fibrinogen and asialofibrinogen, respectively (Table 4.2). The entropy was also negative for all the glycoproteins with a value of ΔS = −44.85, −48.10, −42.56, −45.29, −42.89, −44.02 $J \cdot mol^{-1} \cdot K^{-1}$ for BSM, asialo-BSM, fetuin, asialofetuin, fibrinogen and asialofibrinogen, respectively (Table 4.2).

Table 4.2 : Thermodynamic parameters for the binding of different glycans and glycoproteins to immobilized *Fusarium* lectin at different temperatures: Determined by SPR (Kinetic analysis)

Sugar	ΔH ($kJ \cdot mol^{-1}$)	ΔG* ($kJ \cdot mol^{-1}$)	ΔS ($J \cdot mol^{-1} \cdot K^{-1}$)
Galβ1→3GalNAcα1 →*O*Ser[$]	−22.1	−18.24	−8.27
Asialo-triantennary N-glycan	−44.28	−27.68	−55.73
Asialo-biantennary N-glycan	−43.26	−23.73	−69.03
Asialo-BSM	−72.58	−58.22	−48.10
BSM	−64.68	−51.31	−44.85
Asialofetuin	−64.44	−50.94	−45.29
Fetuin	−52.06	−39.37	−42.56
Asialofibrinogen	−59.89	−46.77	−44.02
Fibrinogen	−49.34	−36.55	−42.89

* Determined at 25 °C
[$] Data was obtained as per Scatchard analysis

BSM contains several O-linked Galβ1→3GalNAcα1→*O*Ser glycans, fetuin has a triantennary structure with ultimate Galβ1→4GlcNAc and three O-linked structure, whereas fibrinogen has biantennary structure with ultimate Galβ1→4GlcNAc (11,12). The *Fusarium* lectin showed very high affinity for desialated glycoproteins in order of asialo-BSM > asialofetuin > asialofibrinogen. The higher affinity of the lectin towards asialo-BSM suggested its preference for O-linked structures Galβ1→3GalNAcα1→*O*Ser. However the observed low affinity for O-linked glycan over N-linked glycans can be explained on the basis of valency since asialo-triantennary is trivalent, asialo-biantennary is bivalent whereas, and O-linked is a monovalent glycan. Asialofetuin showed 5 fold higher affinity for the lectin than asialofibrinogen which can be explained on the basis that asialofetuin possesses three trivalent N-linked as well as three O-linked glycan, a more favorable ligand than fibrinogen which has only bivalent N-linked glycan.

The sialated glycoproteins also bind to *Fusarium* lectin but with 20-100 fold less affinity indicating an unfavorable interaction with neuraminic acid caused by steric hindrance or negative charge. Similar observations were made in lectin from *Sclerotium rolfsii* which showed higher affinity for asialo-BSM and asialofetuin (7). On the contrary lectin from mushroom *Psathyrella velutina* showed higher affinity for sialated glycoproteins than their desialated counterpart (13).

The thermodynamic parameters viz. free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of binding, determined by K_a at different temperatures, are given in Table 4.2. The Van't Hoff plots were linear ($r > 0.9$) for all the glycans and glycoproteins in the temperature range studied (Fig. 4.3).

A decrease in association constants was observed with increasing temperatures indicating negative enthalpy change. This suggested that the binding was exothermic in nature and driven by enthalpy. The values of binding enthalpy (ΔH) were similar for asialo-triantennary (-44.28 $kJmol^{-1}$) and asialo-biantennary (-43.26 $kJmol^{-1}$) pointing towards equal contribution of

hydrogen bonding and van der Walls interactions in the binding and higher affinity is presumably only due to multivalency.

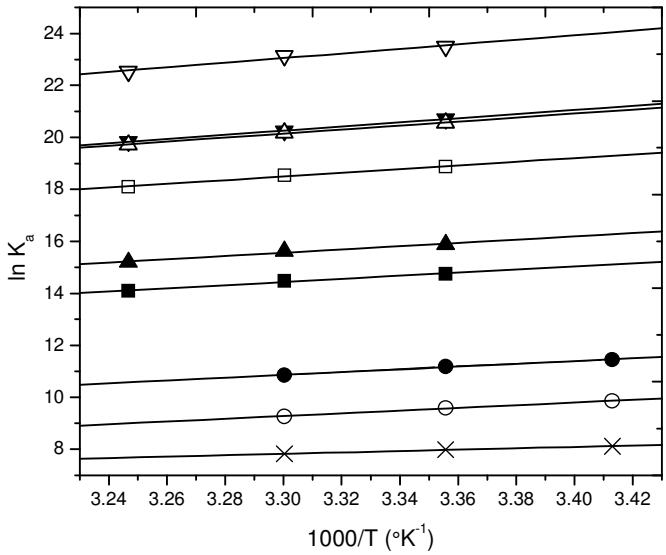

Fig. 4.3 : Van't Hoff plots for the association of various glycans and glycoproteins to *Fusarium* lectin are drawn according to the regression equation. The symbols used are: (●) Asialo-triantennary glycan (n=3, r=0.9970); (○) Asialo-biantennary glycan (n=3 ,r=0.9982); (×) Galβ1→3GalNAcα1→*O*Ser (n=3, r=0.9962); (▽) Asialo-BSM (n=3, r =0.9883); (▼) BSM (n=3, r =0.9997); (▲) Fetuin (n=3, r =0.9901); (△) Asialo-fetuin (n=3, r=0.9979); (■) Fibrinogen (n=3, r =0.9942); (□) Asialo-fibrinogen (n=3, r =0.9957).

Low affinity of O-linked glycan than N-glycan is also manifested in lower enthalpy (ΔH= −22.1 kJ·mol^{-1}) probably caused by loss of some favorable polar interactions. The higher affinity was accompanied by a large and negative change in free energy and enthalpy. However the lower affinity of sialated glycoprotein is also reflected in lower negative enthalpy change than their desialated counterpart, indicating the loss of some favorable enthalpic

contribution. Furthermore, the relatively positive Δ*S* (by the difference of 2-4 $J \cdot mol^{-1} \cdot K^{-1}$) for sialated glycoproteins compared to their desialated counterpart, indicated enhanced nonpolar interactions between sialated glycoproteins and *Fusarium* lectin. Such entropic contribution can be correlated to the rearrangement of water molecules near the binding site and/or loss of structural flexibility of the ligand.

In the present investigation, thermodynamic studies provide an insight in to the role of protein moiety in the lectin-ligand interactions. All the three glycans viz. asialo-triantennary N-glycan, asialo-biantennary N-glycan and O-glycan (Galβ1→3GalNAcα1→*O*Ser), showed lower negative binding enthalpy than their corresponding glycoproteins *viz.* asialofetuin, asialofibrinogen and asialo-BSM. This indicated significant contribution of enthalpy in the free energy change during the binding of these glycoproteins to the *Fusarium* lectin, ultimately leading to their higher affinity. The asialofetuin and asialofibrinogen also showed relatively positive value of binding entropy (Δ*S*) than the asialo-triantennary and asialo-biantennary glycans, respectively, pointing towards some entropic contribution. Asialo-BSM showed relatively negative value of binding entropy than O-glycan Galβ1→3GalNAcα1→*O*Ser, which could be due to compensation of the entropy of O-glycan binding by a much larger enthalpy, during the interaction of asialo-BSM with the lectin. This entropic contribution during binding of these two glycoproteins to the lectin could probably due to some hydrophobic protein-protein interaction or may be compensation for rearrangement of water molecules near the binding site.

Kinetic analysis:

The kinetic analysis of Galβ1→3GalNAcα1→*O*Ser could not be performed due to spikes at the start and end points of injection. The asialo-triantennary glycan (k_1=4.81 × 10^3 $M^{-1}s^{-1}$ and k_{-1}= 0.065 s^{-1}) showed faster association and dissociation rates than asialo-biantennary (k_1=1.89 × 10^2 $M^{-1}s^{-1}$ and (k_{-1}= 0.012 s^{-1}). The association (k_1) and dissociation rate constants (k_{-1})

determined at different temperatures are given in Table 4.3 and 4.4. The Arrhenius plots for all the glycans and glycoproteins were linear (r> 0.9) in the temperature range studied (Fig. 4.4, 4.5 and 4.6).

Table 4.3 : Rate constants and activation parameters for the association process of glycans and glycoproteins to lectin from *Fusarium* sp. (LR11)

Ligand	Association				
	$k_1 \times 10^{-3}$ $M^{-1} s^{-1}$	$E^{\ddagger}_1$ kJ mol^{-1}	$\Delta H^{\ddagger}_1$ kJ mol^{-1}	$\Delta G^{\ddagger}_1$ kJ mol^{-1}	$\Delta S^{\ddagger}_1$ $Jmol^{-1}K^{-1}$
Asialo-triantennary N-glycan	4.81	48.74	46.26	52.06	-19.29
Asialo-biantennary N-glycan	0.189	24.96	22.49	60.01	-125.88
Asialo-BSM	1810	24.58	22.10	38.15	-53.86
BSM	629	14.54	12.07	42.88	-103.4
Asialofetuin	1110	9.16	6.69	47.04	-135.41
Fetuin	8.62	10.45	7.98	47.08	-131.29
Asialofibrinogen	183	11.18	8.70	48.9	-134.9
Fibrinogen	3.15	16.05	13.58	53.01	-132.3

Values of k_1 and $\Delta G^{\ddagger}_1$ are determined at 25 °C.

Table 4.4 : Rate constants and activation parameters for the dissociation process of glycans and glycoproteins to lectin from *Fusarium* sp. (LR11)

Ligand	Dissociation				
	$k_{-1} \times 10^3$ s^{-1}	$E^{\ddagger}_{-1}$ kJ mol^{-1}	$\Delta H^{\ddagger}_{-1}$ kJ mol^{-1}	$\Delta G^{\ddagger}_{-1}$ $Jmol^{-1}K^{-1}$	$\Delta S^{\ddagger}_{-1}$ $Jmol^{-1}K^{-1}$
Asialo-triantennary N-glycan	65	93.02	90.54	79.68	-55.73
Asialo-biantennary N-glycan	12.6	69.28	66.75	83.69	-56.84
Asialo-BSM	0.11	96.5	94.03	95.6	-5.3
BSM	0.63	78.70	76.23	93.89	-59.28
Asialofetuin	1.31	73.7	71.23	98.08	-90.12
Fetuin	1.08	62.40	59.93	86.50	-89.18
Asialofibrinogen	1.15	69.95	67.48	93.94	-88.81
Fibrinogen	1.23	65.62	63.15	89.61	-88.8

Values of k_{-1} and $\Delta G^{\ddagger}_{-1}$ are determined at 25 °C.

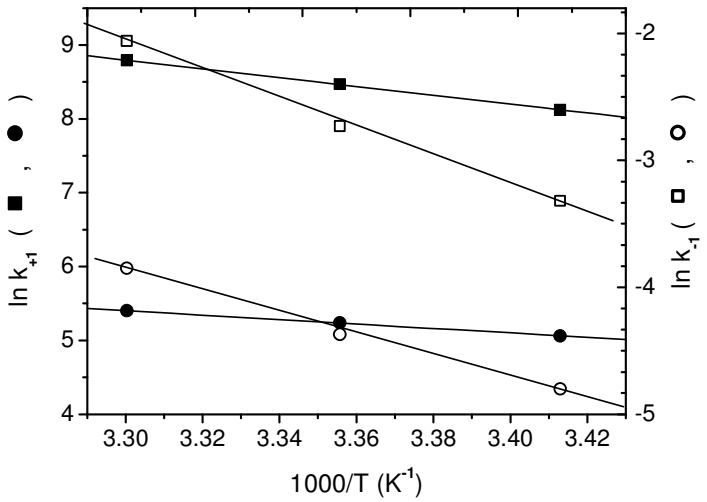

Fig. 4.4 : Arrhenius plots for the association and dissociation kinetics of various glycans to *Fusarium* lectin. The symbols used are: association of (■)asialo-triantennary glycan; and (●) asialo-biantennary glycan; dissociation of (□) asialo-triantennary glycan and (○) asialo-biantennary glycan glycans.

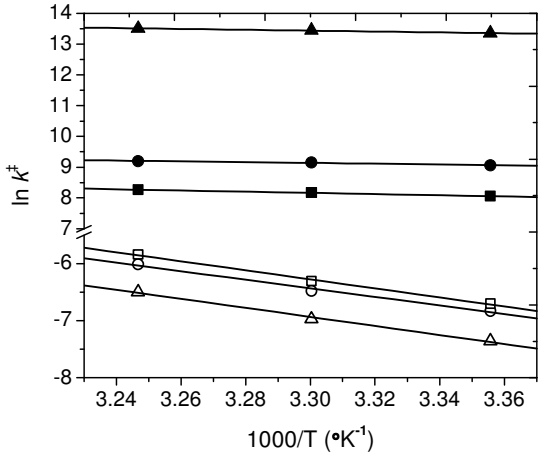

Fig. 4.5 : Arrhenius plots for the association and dissociation kinetics of sialated glycoproteins to *Fusarium* lectin. The symbols used are: : (▲) association of BSM (n=3, r =0.9918); (△) dissociation of BSM (n=3, r =0.9977); (●) association of fetuin (n=3, r =0.9876); (○) dissociation of fetuin (n=3, r =0.9962; (■) association of fibrinogen (n=3, r =0.9986) and (□) dissociation of fibrinogen (n=3, r =0.99877);

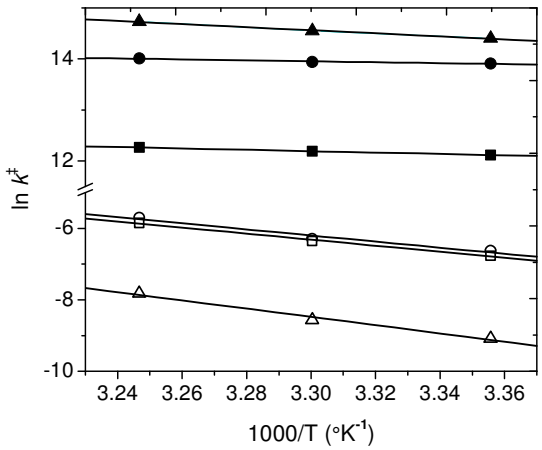

Fig. 4.6 : Arrhenius plots for the association and dissociation kinetics of desialated glycoproteins to *Fusarium* lectin. The symbols used are: : (▲) association of asialo-BSM (n=3, r =0.9980); (△) dissociation of asialo-BSM (n=3, r =0.9939); (●) association of asialofetuin (n=3, r =0.9877); (○) dissociation of asialofetuin (n=3, r =0.9966; (■) association of asialofibrinogen (n=3, r =0.9999) and (□) dissociation of asialofibrinogen (n=3, r =0.9978).

The glycoproteins showed significantly higher association and slower dissociation rate constants than glycans. Asialo-fetuin, fetuin, asialo-fibrinogen and fibrinogen showed similar dissociation rates with a marginal difference in the association rates, resulting in difference in affinity. However, BSM and asialo-BSM showed higher association and lower dissociation rates compared to other glycoproteins resulting in their higher affinity.

The association rate constants are of several orders of magnitude slower than diffusion controlled reactions and the kinetics of ligand binding to lectins is qualitatively consistent with a single-step binding mechanism (Table 4.3), and can be depicted by the equation:

$$P + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PL \quad ; \quad K_a = k_1/k_{-1}$$

Bimolecular association rate constants which are slower than the diffusion controlled process have also been observed for lectins from *Ricinus communis* (14), soybean (15) and *Artocarpus* (16).

Generally, when the second order rate constants for the ligand (L) binding to a protein (P) are slower by several orders of magnitude than those seen in diffusion controlled reactions, the binding is presumed to involve a putative intermediate complex PL*, which then isomerizes into a final complex PL.

$$P + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PL^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PL$$

Where $K_1 = k_1/k_{-1}$ and $K_2 = k_2/k_{-2}$. Since the first step is too rapid to measure by SPR, it is possible that we are observing only the second step. The agreement between kinetically determined values of association constants (k_1/k_{-1}) and changes in enthalpies with those determined by Scatchard analysis indicates that the K_a and the enthalpy changes are related to the total binding process and not to due to any intermediate that contributes appreciably to these parameters for the saccharide binding. Linearity of Arrhenius plots, however, also rules out to a great extend, the formation of such an intermediate(s) and the occurrence of dramatic conformational changes in the lectin molecule at

least in the temperature range studied. Thus it can be concluded that the binding of glycans and glycoproteins to the *Fusarium* lectin is consistent with a single step bimolecular association reaction which is of several orders of magnitude slower than diffusion controlled reaction.

Asialo-triantennary glycan binds 20 times faster than asialo-biantennary glycan and the activation energy ($E^{\ddagger}_1$) for the association process of the asialo-triantennary (48.7 kJmol^{-1}) is higher than asialo-biantennary (24.96 kJ mol^{-1}), indicating that large amounts of energy has to be expended for the binding to take place. The entropy of activation for association process with asialo-triantennary glycan (-19.29 J mol^{-1} K^{-1}) is small, indicating that association process does not involve a highly ordered transition state. This suggests that the sugar can approach the binding pocket in several ways, leading to faster association. The entropy of activation for association process with asialo-biantennary glycan is comparatively high (-125.9 J mol^{-1} K^{-1}) indicating a highly ordered transition state is implied in the binding to the lectin.

The value of K_a determined by k_1/k_{-1}, can increase either by increase in k_1 or decrease in k_{-1}. For most protein-ligand interactions decrease in dissociation rate constants (k_{-1}) has been shown to be responsible for increased binding affinity and not increase in the association rate constants (k_1) (17). In the present studies, higher association rate constant was observed for binding of asialo-triantennary glycan to the lectin than asialo-biantennary. Moreover, the slower association rate constant and higher entropy of association process appears to be main factors responsible for lower affinity of asialo-biantennary glycan than asialo-triantennary.

Similar dissociation rate constants (k_{-1} = 1-3 $\times$ 10^{-3} s^{-1}) were observed with asialofetuin, asialofibrinogen, fetuin and fibrinogen despite of differences in association constants, suggest that different association rates are solely responsible for the differences in the affinity of these glycoproteins. However, the higher affinity of BSM and asialo-BSM than other glycoproteins is due to their faster association as well as slow dissociation. The activation energy of

the association process ($E^{\ddagger}_1$) was approximately half for glycoproteins as compared to glycans, indicating that comparatively low amount of energy has to be expended for the binding to take place. Glycoproteins like, BSM, fetuin, asialofetuin, fibrinogen and asialofibrinogen showed high negative entropy of association ($-\Delta S^{\ddagger}_1 > 100$ J·mol^{-}1·K^{-1}) suggesting that the association process involves a highly ordered transition state. The comparatively low entropy of association for asialo-BSM, ($-\Delta S^{\ddagger}_1 = 53.86$ J·mol^{-1}·K^{-1}), most potent ligand, showed that it can approach the binding pocket in the lectin in several ways leading to higher affinity. This is in contrast with peanut agglutinin (18), where a highly ordered transition state has been implicated in an entropically driven binding reaction ($-\Delta S^{\ddagger}_1 = 160$ J·mol^{-1}·K^{-1}).

In conclusion, the present studies show that the higher affinity of *Fusarium* lectin for glycoproteins is accompanied by enthalpic as well as entropic contribution. In addition higher association and lower dissociation rates as well as low activation energy of association process contribute to its higher affinity for glycoprotein compared to glycans.

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CHAPTER : 5

DENATURATION AND UNFOLDING OF *FUSARIUM* LECTIN

SUMMARY

The conformational stability of *Fusarium* lectin was determined with chemical, thermal and pH denaturants. Equilibrium unfolding with guanidine thiocynate showed that the values of $D_{1/2}$ (0.49 M) and ΔG^{H_2O} (5.24 kJ/mol) were lowest at pH 12. The maximum conformation stability of *Fusarium* lectin was observed at pH 8.0 near its pI. At pH 12, the ΔG^{H_2O} and $D_{1/2}$ values of urea induced unfolding was higher than that of guanidine thiocynate. Curves of fraction unfolded (f_U) obtained with fluorescence and CD measurements overlapped at all the pH studied. Moreover, the denaturation of the lectin was irreversible at higher denaturant concentration. At acidic pH, *Fusarium* lectin showed a flexible tertiary structure with pronounced secondary structure and retention of its hemagglutinating activity. Thermal denaturation did not result in aggregation or precipitation of the protein even at high temperatures. ANS binding studies revealed no exposure of hydrophobic patches.

INTRODUCTION

Specific biological functions of proteins emerge directly from their unique and highly individualistic three-dimensional structure, attained in a very short time after their synthesis. The three dimensional structure of a protein is held together by non-covalent interactions *viz*. hydrogen bonds, ionic interactions, hydrophobic interactions, van der Waals forces and covalently by disulfide linkages. Conditions which disturb these stabilizing forces affect the native conformation of the protein by changing a majority of its physical properties apart from its biological activity. The extent and balance between different stabilizing forces, studied under various denaturing conditions, is interpreted in terms of conformational stability. Determination of the conformational stability of a protein is critical for understanding the physical interactions that stabilize the protein. Determination of the stability of a protein is generally based on the analysis of denaturant or thermally induced unfolding transition measured either spectroscopically or calorimetrically (1-5).

The folding pathway of a proteins, sometimes, involves intermediate states and hence it is important to study such partially folded conformations, to understand the principles governing protein folding/unfolding (6,7). Such stable intermediates have been identified and characterized for several proteins using modern sensitive techniques *viz.* spectroscopy and NMR (8).

The recognition of carbohydrate moieties by lectins, has important applications in a number of biological processes such as cell-cell interaction, signal transduction, cell growth and differentiation (9). The functionality of lectin molecules depends on the specific carbohydrate recognition domain, a part of the three dimensional structure of the protein. A perturbed lectin structure does not allow it to interact with the carbohydrate ligands. Thermodynamic parameters such as ΔG, ΔH and ΔS, allow to understand the capability of different forces, holding the three dimensional structure of protein.

This Chapter describes studies on the conformational stability of *Fusarium* lectin through chemical, thermal and pH induced denaturation.

MATERIALS

Urea, guanidine thiocynate and 8-anilino-1-naphthalene sulfonate (Sigma Chemical Co., St. Louis, MO, USA) were used. All other chemicals used were of analytical grade.

METHODS

Protein determination

Protein concentrations were determined according to Bradford *et al.* (10) using BSA as standard.

Purification of *Fusarium* lectin

Cultivation of *Fusarium* sp. (LR11) and purification of lectin was carried out as described in Chapter 2.

Buffers and solutions

The buffers used at different pH: Glycine-HCl (pH 2-3), sodium acetate (4-5), sodium citrate-phosphate (5), sodium phosphate (6-7), Tris-HCl (8-9) and glycine-NaOH (10-12). The urea and Gdn-HSCN stock solutions were prepared fresh on the day of use as described by Pace *et al.* (11).

Equilibrium unfolding studies

Equilibrium unfolding as a function of urea, Gdn-HSCN (guanidine thiocynate), pH and temperature was monitored by fluorescence. For chemical denaturation, the protein (25 μg/ml) was incubated in presence of various concentrations of the denaturant in 50 mM buffers of various pH, at 25 °C for 24 h. In the thermal unfolding experiments, the spectra were recorded 15 min after the desired temperature was attained. The intrinsic tryptophan fluorescence spectra of the protein were recorded on a Perkin Elmer LS-50B spectrofluorimeter, equipped with Julabo F25 water bath. The protein was excited at 280 nm using a cell of 1.0 cm pathlength and both excitation and emission slit widths were set at 7 nm. CD spectra were recorded on a computer interfaced JASCO J-715 spectropolarimeter using a cylindrical quartz cell of 1 mm.

Refolding of *Fusarium* lectin

Two hundred microliters aliquot was taken out from the samples treated with different concentrations of Gdn-HSCN, diluted 10 times with 50 mM buffer of the same pH at which the unfolding was done. After 4 h, the fluorescence spectra and hemagglutinating activity of the original (treated with Gdn-HSCN) as well as diluted samples were recorded. Protein samples without Gdn-HSCN treatment under identical conditions were taken as control.

The renaturation of thermally denatured protein was followed by cooling the heated samples to 20 °C, removing any particulate matter by centrifugation, and then monitoring the fluorescence spectra and the activity.

Hydrophobic dye binding studies

A stock solution of ANS was prepared in methanol and the concentration was measured using an extinction coefficient of 5000 $M^{-1} \cdot cm^{-1}$ at 350 nm (12). ANS emission spectra were recorded in the range of 400-550 nm with excitation at 375 nm using slit widths of 5 nm for emission and excitation monochromators. Changes in the ANS fluorescence, induced by the binding to the lectin, were recorded at constant concentration of protein (2 μM) and ANS (50 μM). The spectrum of ANS alone in buffer was subtracted from the protein-ANS spectrum to yield the final spectrum.

Light scattering studies

Rayleigh light scattering experiments were carried out with the spectrofluorimeter to follow protein aggregation at different pH with increasing temperature. Both excitation and emission wavelength were set at 400 nm and the time dependent changes in scattering intensity were followed.

Gel permeation-HPLC

Lectin samples (100μg in 100μl), pre-incubated for 24h at different pH (at 25°C) were injected onto a Protein-PAK SW300 HPLC gel permeation column (300 x 7.8 mm) connected to a Waters HPLC system, at a flow rate of 0.5 ml/ min. The standard molecular mass markers run in the buffer were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa).

Data analysis

The denaturation curves were plotted, with the fluorescence intensities at the emission λ_{max} of native protein and denatured protein, against denaturant concentration and temperature, and further analysis of the data was performed as described by Pace *et al.* (11). From the denaturation curves, a two state $F \rightleftharpoons U$ unfolding mechanism was assumed, and consequently, for any of the points, only the folded and unfolded conformations were present at significant

concentrations. Thus, if f_F and f_U represent the fraction of protein present in the folded and unfolded conformations, respectively then $f_F + f_U = 1$

f_U was calculated using the following equation

$$f_U = (F_F - F_0) / (F_F - F_U) \quad (1)$$

where, F_F is the fluorescence intensity of completely folded or native protein, F_0 is the observed fluorescence intensity at point of denaturant concentration or temperature, F_U is fluorescence intensity of the completely denatured or unfolded protein.

For a two state F⇌U unfolding mechanism, the equilibrium constant K and ΔG_U, the free energy of unfolding was calculated using Equation 2 and 3 respectively.

$$K = f_U / (1 - f_U) \quad (2)$$

$$\Delta G_U = - RT \ln K \quad (3)$$

where, R is the gas constant, and T is the absolute temperature. It is assumed that the free energy of unfolding, ΔG_U, has a linear dependence on the concentration of the denaturant [D].

$$\Delta G_U = \Delta G^{H_2O} + m[D] \quad (4)$$

ΔG^{H_2O} and m are therefore the intercept and the slope respectively, of the plot of ΔG_U versus [D]. ΔG^{H_2O} corresponds to the free energy difference between the folded and unfolded states in the absence of any denaturant and m is a measure of the cooperativity of the unfolding reaction. The concentration of denaturant at which the protein is half unfolded (when $\Delta G_U = 0$) is given by $D_{1/2}$ and from Equation 4, $\Delta G^{H_2O} = - m D_{1/2}$.

The data from the thermal unfolding curves were obtained under the same conditions as those for denaturant unfolding curves. Values of f_U, K and ΔG_U were calculated using Equations 1, 2 and 3. The midpoint of thermal (T_m) denaturation was obtained as the temperature at which $\Delta G_U = 0$ from the plot of ΔG_U versus T. The slope of such a plot at T_m yielded ΔS_m, the change in entropy. The enthalpy change for unfolding at T_m, ΔH_m, was calculated using the equation,

$$\Delta H_m = T_m \Delta S_m \quad (5)$$

ΔC_p, the change in heat capacity that accompanies protein unfolding was obtained from the slope of the plot of ΔH_m versus T_m, where T_m was varied as a function of Gdn-HSCN concentration. ΔG_U at 25°C was calculated using equation,

$$\Delta G(T) = \Delta H_m(1- T/T_m) - \Delta C_p[(T_m - T) + T \ln(T/T_m)] \quad (6)$$

RESULTS AND DISCUSSION

A protein molecule has a biological activity conferred by its three dimensional structure. Perturbation of any of the forces holding the protein three dimensional structure results in loss of its structure and biological functionality. Such perturbants provide a way to estimate conformation stability of a protein in comparison with others. Denaturation of *Fusarium* lectin was followed by monitoring the changes in physical properties of the protein *viz.* intrinsic fluorescence and circular dichroism. Tryptophan fluorescence has been used to study protein denaturation for several proteins (13-15), which is greatly influenced by the environmental factors such as polarity, pH, temperature and composition of solvent (16).

Chemical denaturation

Denaturation of *Fusarium* lectin with urea and Gdn-HSCN at various pH was monitored by following the changes in intrinsic fluorescence of the protein on excitation at 280 nm. Considerable change in fluorescence spectra, in presence of urea, was observed only at pH 12, whereas it was observed at all the pH studied, with Gdn-HSCN. At higher concentration of Gdn-HSCN, the fluorescence quenching was upto 80 %, whereas it was 32 % in case of urea (Fig. 5.1 and 5.2). In the presence of denaturant, a decrease in fluorescence intensity (Fig. 5.1A) accompanied with red shift in λ_{max} towards 356 nm (Fig. 5.1B) was observed, a characteristic for the fluorescence spectra of Trp residues in an aqueous environment, suggesting unfolded state of the lectin. This conclusion is also supported with the appearance Tyr peak at 307 nm. It has been suggested that under denaturing conditions the energy transfer from Tyr to Trp residues does not occur any more (17). The decrease in fluorescence intensity may be due to the denaturation of protein and can be explained on the basis of increase in the polarity of tryptophan environment, as it becomes fully exposed to the solvent. It has been suggested that the excited singlet states of tryptophan interact with water molecules to form

excited state complexes and such a process competes with the radiative relaxation and leads to diminution of the fluorescence intensity (18-21).

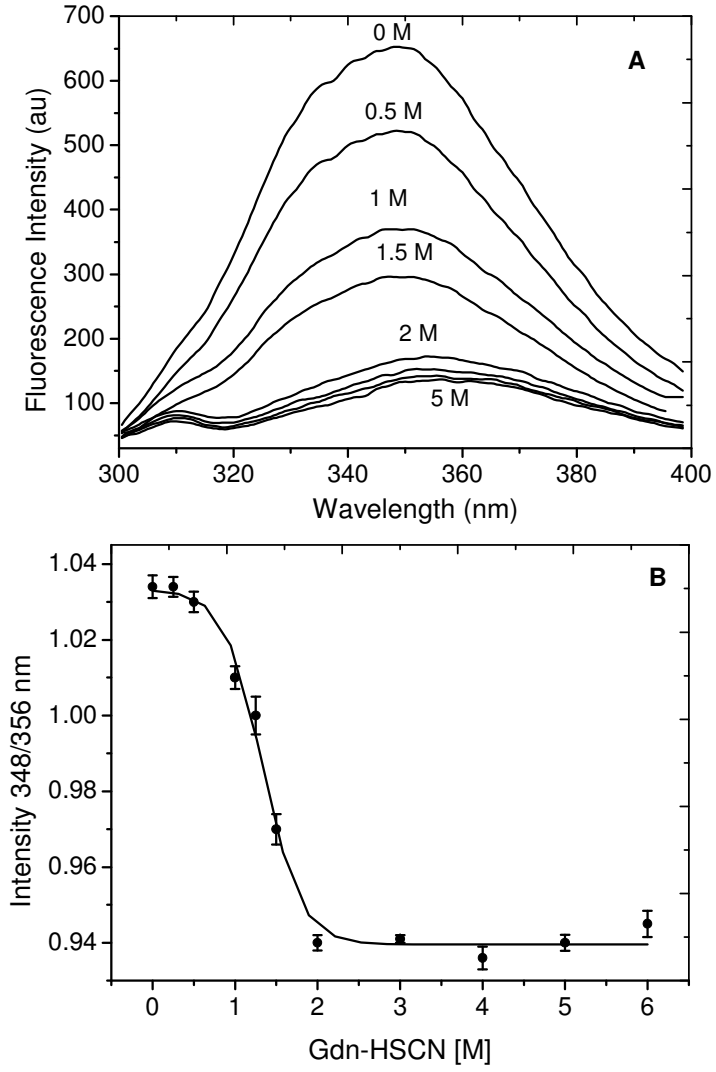

Fig. 5.1 : Equilibrium unfolding of *Fusarium* lectin in the presence of Gdn-HSCN at pH 8.0. (A) Fluorescence spectra (B) Denaturation curve obtained by plotting ratio of fluorescence intensities at wavelengths 348 and 356 nm as a function of Gdn-HSCN concentration. The spectra were recorded after 24 h incubation of the lectin in different concentration of Gdn-HSCN at 25 °C.

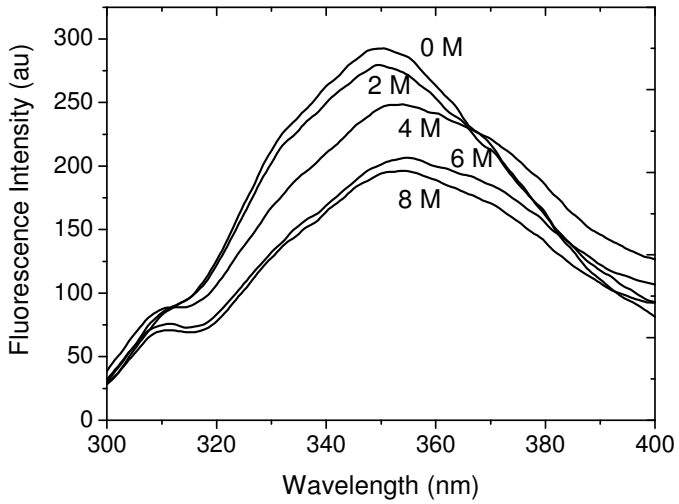

Fig. 5.2 A : Fluorescence spectra of *Fusarium* lectin in the presence of varying concentration of urea at pH 12

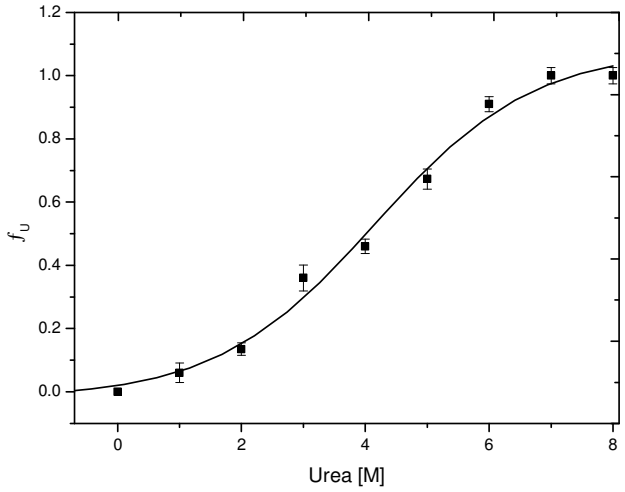

Fig. 5.2 B : f_U curves for urea induced denaturation. The fraction unfolded (f_U) from fluorescence were calculated using Eq. 1 and plotted as a function of urea concentration at pH 12.0

The denaturation of *Fusarium* lectin was also monitored by following the changes in the far-UV CD spectra (Fig. 5.3). The denaturation curves were prepared by plotting θ_{220} as a function of denaturant concentration and the f_U was calculated using Equation 1. The f_U plots obtained from CD data were in agreement with those obtained by fluorescence spectroscopy (Fig. 5.4). The majority of the proteins exhibit a single step denaturation curve and so are assumed to follow a two-state N⇌D mechanism in which only the native state and denatured states are present at significant concentrations in the transition region (11,22,23). Superimposition of f_U curves, obtained by fluorescence and CD measurements for Gdn-HSCN induced denaturation, supports this assumption (Fig. 5.4).

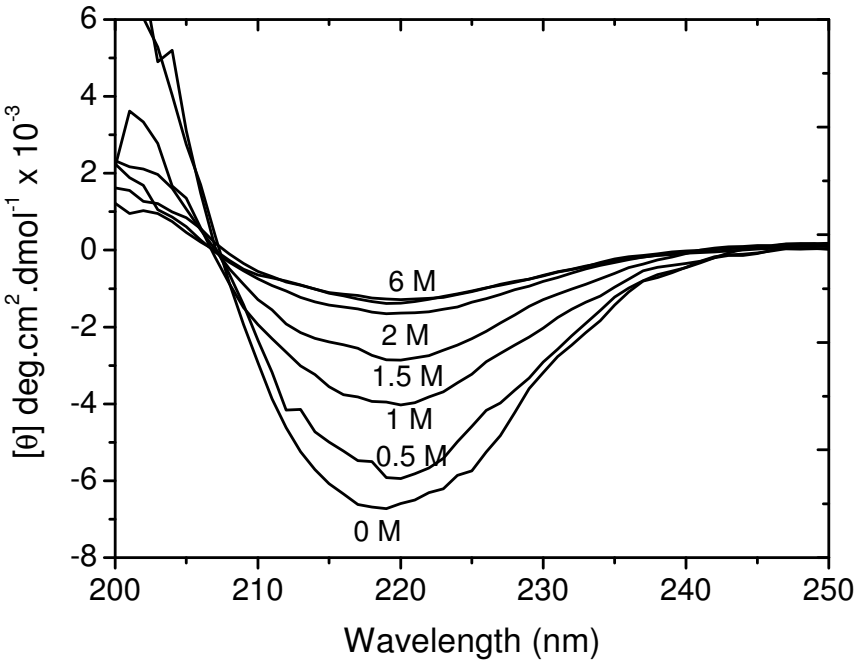

Fig. 5.3 : Far-UV CD spectra of *Fusarium* lectin in the presence of Gdn-HSCN at pH 8.0. Concentration of denaturant is indicated in figure.

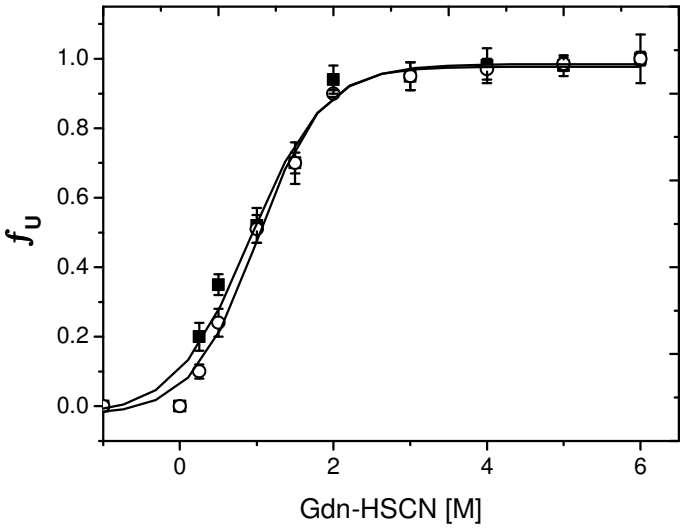

Fig. 5.4 : f_U curves for Gdn-HSCN induced denaturation. The fraction unfolded (f_U) from fluorescence (■) and far-UV CD (○) were calculated using Eq. 1 and plotted as a function of Gdn-HSCN concentration at pH 8.0

The data obtained from fluorescence spectra was used to prepare the denaturation curves, which were then analyzed to obtain ΔG_U, using Equations 1, 2 and 3. The ΔG_U was plotted as a function of denaturant concentration to obtain $D_{1/2}$ and ΔG^{H_2O} (Fig. 5.5). The change in free energy (ΔG), associated with *Fusarium* lectin unfolding, varied linearly ($r > 0.99$) with the increase in urea and Gdn-HSCN concentrations. The values of $D_{1/2}$ (3.88 M), ΔG^{H_2O} (9.38 KJ/mol) and $-m$ (2.4 kJ/mol/deg) were obtained for urea denaturation at pH 12 (Table 5.1). The denaturation was observed at lower concentrations of Gdn-HSCN at pH 12 than at pH 6, 8 and 10 and the value of ΔG^{H_2O} (5.24 kJ/mol) and $D_{1/2}$ (0.49 M) were lowest at pH 12 (Table 5.2). Moreover, the of $D_{1/2}$ value obtained with Gdn-HSCN (0.49 M) at pH 12 was approximately eight times lower than that with urea. The maximum value of ΔG^{H_2O} (8.45 kJ/mol) with Gdn-HSCN was obtained at pH 8.0. In general, proteins are most stable at their isoelectric pH since the electrostatic interactions among the charged groups will tend to favor the maximum conformational stability

occurring near the pI (24). Similar observations also have been made in some other proteins in presence of chaotropes (25,26).

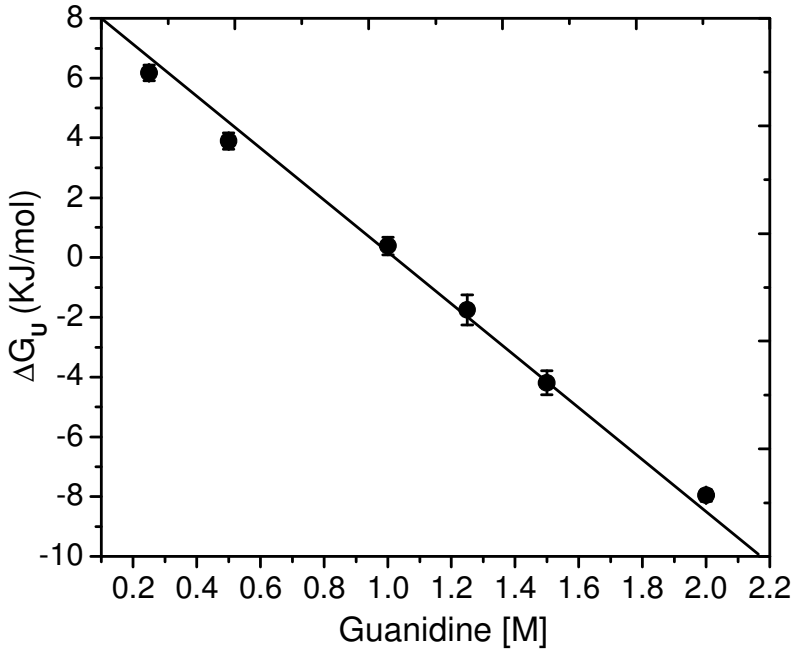

Fig. 5.5 : ΔG_U as a function of Gdn-HSCN concentration at pH 8.0. ΔG_U was calculated using Equation 3, intercept and slope of the graph equals to ΔG^{H20} and *m,* respectively.

Table 5.1 : Parameters for urea denaturation of *Fusarium* lectin[a]

pH*	ΔG^{H_2O} (kJ/mol)	$-m$ (kJ/mol/deg)	$D_{1/2}$ M
12	9.38	2.4	3.88

[a] The values represent the mean of three independent sets of experiments with SD of less than 10%.

* At pH 6, 8 and 10 no change in fluorescence spectrum was observed.

$D_{1/2}$, m, and ΔG^{H_2O} values were obtained with linear extrapolation method from plots of ΔG_U as a function of urea concentration using Eq. (4).

Table 5.2 : Parameters for guanidine thiocynate denaturation of *Fusarium* lectin[a].

pH	ΔG^{H_2O} (kJ/mol)	$-m$ (kJ/mol/deg)	$D_{1/2}$ M
6	7.46	7.86	0.95
8	8.45	8.05	1.05
10	7.55	7.63	0.99
12	5.24	10.7	0.49

[a] The values represent the mean of three independent sets of experiments with SD of less than 10%.

$D_{1/2}$, m, and ΔG^{H_2O} values were obtained with linear extrapolation method from plots of ΔG_U as a function of urea concentration using Eq. (4).

Influence of pH revealed that the lectin showed minimum stability at pH 12. Higher pH causes ionization of tyrosine and histidine residues, which are generally packed in the hydrophobic interior of the protein. At high pH, therefore, ionization of these groups make the protein more susceptible to urea and Gdn-HSCN induced denaturation. The *Fusarium* lectin showed marginally lower value of ΔG^{H_2O} (6-9 kJ/mol) as compared to other proteins. The conformational stability of almost all naturally occurring globular proteins has been observed to be in the range of 20- 60 kJ/mol (24). In addition, no ANS binding and protein aggregation was observed in chemical denaturation and the unfolded lectin did not exhibit hemagglutinating activity. Refolding was measured as the extent of reappearance of the original spectra and recovery of the hemagglutinating activity. However, no considerable refolding was observed in denatured *Fusarium* lectin.

pH dependent denaturation

Tryptophan fluorescence, which is influenced by environmental factors like polarity, composition, pH and temperature of the solvent, has been used to study protein denaturation of several proteins (13-16). Influence of pH on denaturation of *Fusarium* lectin showed quenching at lower pH without any shift in the λ_{max} (Fig. 5.6). The λ_{max} at 348 nm, indicated relatively exposed tryptophan residues since completely exposed tryptophan has a λ_{max} between 355-360 nm, characteristic of totally unfolded protein (18,27-29). Thus the acid induced unfolding of *Fusarium* lectin does not cause Trp to come in direct contact with water. However, the Trp fluorescence and its dependence on pH agrees well with the results of fluorescence studies performed with some other proteins and model substances (27,30). Emission intensity observed at 348 nm remained constant over pH 6-10 while it gradually decreased below pH 5. The maximum decrease in fluorescence emission intensity was 95 % at pH 2.

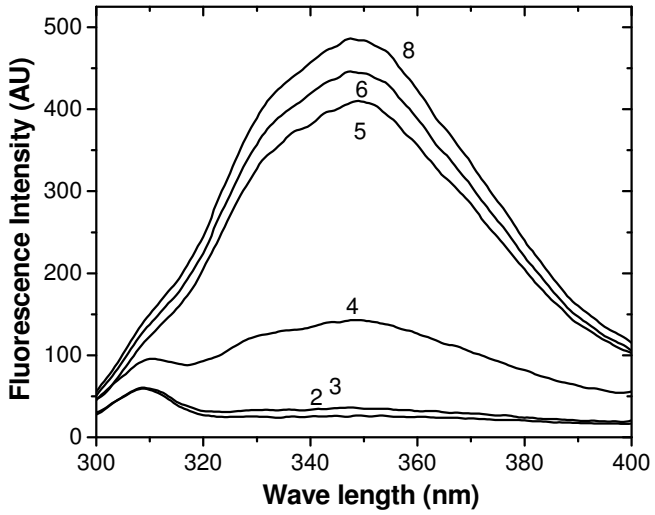

Fig. 5.6 : Fluorescence spectra of *Fusarium* lectin at different pH. The lectin was incubated at different pH (as denoted by numbers) for 24 h, at 25 °C.

In most of the proteins the Trp fluorescence dominates the Tyr fluorescence even in the proteins containing more Tyr than Trp. This is caused by relatively high absorbance of Trp residues and relatively low fluorescence efficiency of most Tyr residues, and often by an efficient transfer of excitation energy from Tyr to Trp (27,30). Furthermore, it has also been shown that the observed reduction of fluorescence at low and high pH values cannot be explained simply in terms of partial denaturation of the protein in acid or alkaline environment. The reduction of fluorescence intensity at low pH values can be caused by quenching of fluorescence by hydrogen ion (acid quenching) and neutralization of COO^- groups on acidic amino acid in vicinity of fluorophores (30).

The far-UV CD spectra of the native and acid perturbed lectin showed that both the forms possess considerable secondary structure (Fig. 5.7). The molar ellipticities at 220 nm were 7.46, 7.47 and 8.13 deg·cm^2·dmol^{-1} at pH 2, 4 and 6 respectively.

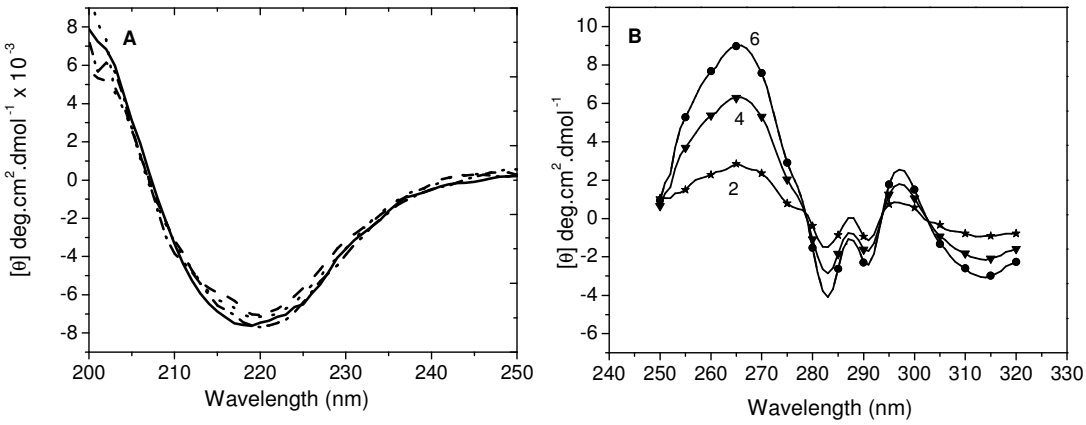

Fig. 5.7 : pH dependent denaturation of *Fusarium* lectin. (A) far-UV CD spectra. The symbols used are: (—) pH 2, (---) pH 6, (····) pH 8, and (-·-·-) pH 12. (B) near-UV CD spectra (pH denoted by numbers). The spectra were recorded at different pH after 24 h incubation at

The lectin retained approximately 92% of its secondary structure between pH 2-4. On the other hand, the near-UV CD spectrum showed distinct positive peaks at 266 and 296 nm and a negative peak 283 nm for phenylalanine,

tryptophan and tyrosine, respectively. The molar ellipticity in the near-UV region at pH 2 was lower than that observed at pH 6, suggesting that the low pH provides a more flexible environment for the aromatic residues (31).

8-Anilino-1-naphthalene sulfonate (ANS) does not fluoresce in aqueous solution but does in organic solvents or when exposed to hydrophobic environment. ANS does not bind to native or completely unfolded polypeptide chain but it binds to intermediates formed during the unfolding. ANS bound to *Fusarium* lectin between pH 2-4 with an increase in the fluorescence intensity accompanied by blue shift in the emission maximum (Fig. 5.8). The fluorescence intensity of ANS was 6.8 and 3.8 times higher at pH 2 and 4, respectively from those between pH 6-10. *Fusarium* lectin showed considerable secondary structure between pH 2-4, with an exposure of large hydrophobic patches to the solvent.

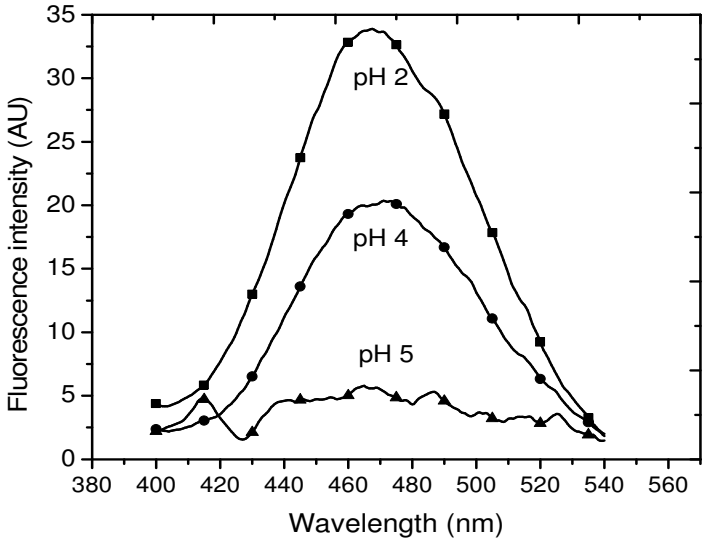

Fig. 5.8 : ANS binding to *Fusarium* lectin. Change in ANS fluorescence (50 μM) was recorded in the presence of *Fusarium* lectin (2 μM) at different pH (as denoted by numbers) on excitation at 375 nm.

Gel permeation of the *Fusarium* lectin showed single peak corresponding to molecular mass of 26 kDa at pH 6. However, at pH 3 and 4, no change in elution profile was observed, which excluded the possibility of monomerization or aggregation of the lectin (Fig. 5.9). In the hemagglutination assays, the lectin was found to have hemagglutinating activity in a broad range of pH varying from 2-12.

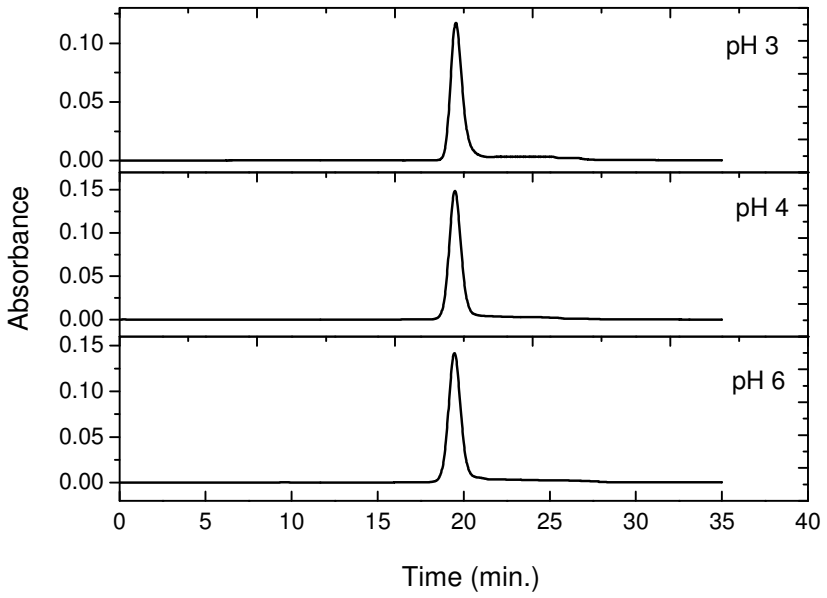

Fig. 5.9 : Gel Permeation-HPLC of *Fusarium* lectin at different pH. The lectin (100 µg) was incubated for 24 h at respective pH and 25 °C, injected on HPLC (Waters Corporation) gel permeation column (Protein-PAK SW300, 300×7.8 mm).

The molecular mass of acid denatured lectin suggested its presence in the dimeric form. At lower pH, it retained approximately 92 % and 70 % of its secondary and tertiary structure respectively. The ANS binding data suggested that it was highly solvent exposed. This type of structure of *Fusarium* lectin does not fit into the classical concept of molten globule as the

latter represents a compact native like structure of the protein with pronounced secondary structure devoid of any rigid tertiary and quaternary structure. The acid perturbed state of *Fusarium* lectin is represented by its dimeric form with pronounced secondary structure and its hemagglutinating activity. Whereas, the molten globule state, reported for dimeric, peanut lectin (32) and galectin-1 (14), are represented by monomeric carbohydrate binding entities.

The pH induced unfolding of the proteins is achieved by protonation or deprotonation of relatively few discrete sites (-OH, -COOH and $-NH_2$) (33). Thus, unlike temperature and chaotropes, pH induced unfolding of the proteins is achieved by perturbance of few residues and a complete unfolding of the protein cannot be expected. In the present studies, the acid induced loss of tertiary structure of *Fusarium* lectin can be due to perturbation of the ionic and/or hydrogen bond interactions. However, this perturbation does not appear to affect the interactions involved in hemagglutination activity.

Thermal denaturation in presence of low concentration of Gdn-HSCN

Incubation of the *Fusarium* lectin at various temperatures upto 80 °C resulted in fluorescence quenching without any shift in λ_{max} indicating that the lectin retains its conformational integrity even at high temperature. Moreover, the far-UV CD spectra at 80 °C also did not show any loss of ellipticity. Hence, thermal denaturation was carried out in the presence of low concentrations of Gdn-HSCN (0-0.4 M), which did not result in unfolding of the lectin at low temperatures. Thermal denaturation in the presence of Gdn-HSCN resulted in fluorescence quenching with a shift in emission λ_{max} towards 356 nm (Fig 5.10) as well as loss of ellipticity in far-UV CD spectrum (Fig. 511). The fluorescence data was used to prepare the denaturation curves at varying concentrations of Gdn-HSCN as a function of temperature; and the data was analyzed using Equations 1-3 (Fig. 5.12). ΔG_U was plotted against T to obtain T_m and ΔS_m (Fig. 5.13). The change in enthalpies (ΔH_m), determined at T_m for different Gdn-HSCN concentrations were plotted as a function of T_m (Fig. 5.14). The slope of this plot yielded ΔC_p (3.95 kJ/mol/deg), which was then

used to calculate ΔG (at 25 °C) from the thermal denaturation curves at different concentrations of Gdn-HSCN (Table 5.3). The value of free energy, ΔG, was approximately half of that required for chemical induced denaturation. The $T_{m,}$ ΔH_m and ΔG of thermal unfolding showed a decrease with the increase in the Gdn-HSCN concentration.

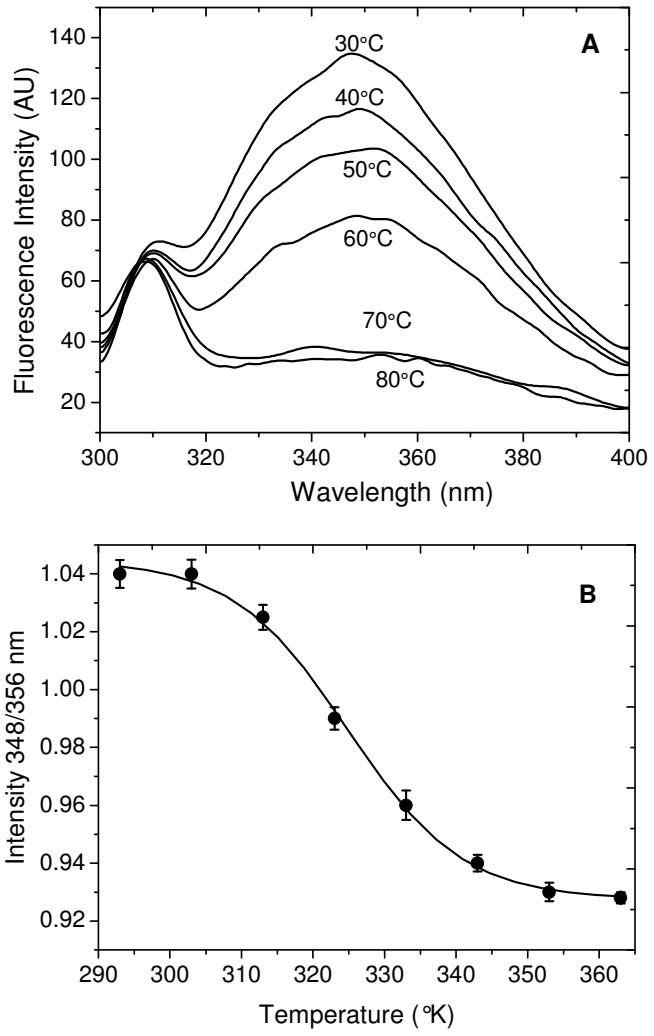

Fig. 5.10 : Thermal unfolding of *Fusarium* lectin in the presence of 0.1 M Gdn-HSCN at pH 8.0. (A) Fluorescence spectra (B) Denaturation curve obtained by plotting ratio of fluorescence intensities at wavelengths 348 and 356 nm as a function temperature. The spectra were recorded after 15 min incubation of the lectin at different temperature.

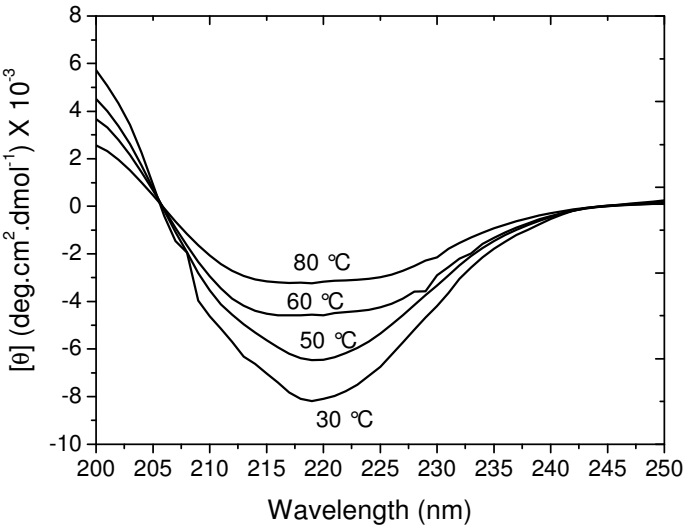

Fig. 5.11 : Far-UV CD spectra of *Fusarium* lectin in the presence of 0.4 Gdn-HSCN at various temperature (as denoted in the figure).

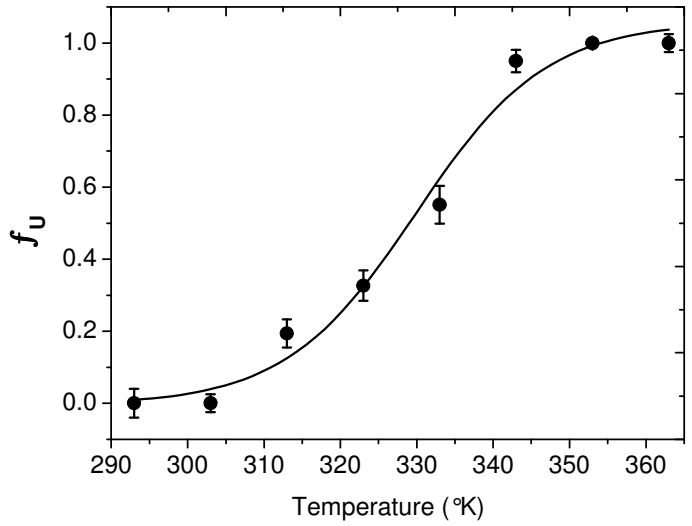

Fig. 5.12 : f_U curves for thermal denaturation in the presence of 0.1 M Gdn-HSCN at pH 8.0.. The fraction unfolded were calculated using Eq. 1 and plotted as a function of temperature.

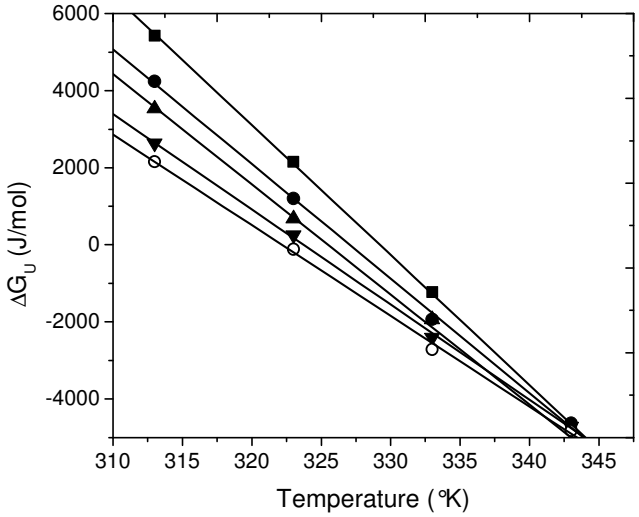

Fig. 5.13 : ΔG_U as a function of temperature. ΔG_U was calculated using Equations (1)-(3) from the thermal denaturation curves obtained at pH 8 in the presence of Gdn-HSCN (■) 0.05 M, (●) 0.1 M, (▲) 0.2 M, (▼) 0.3 M and (○) 0.4 M.

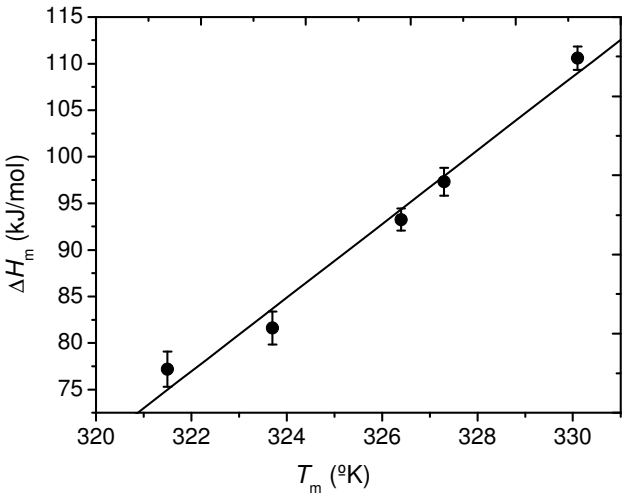

Fig. 5.14 : Determination of ΔC_p. Thermal denaturation experiments were performed in the presence of different concentrations of Gdn-HSCN at pH 8.0 to vary T_m. T_m, as the transition midpoint and ΔS_m, as the slope were obtained from the plots of ΔG_U versus T. ΔH_m was determined at each value of T_m using Equation (5).

Table 5.3 : Parameters for thermal denaturation of *Fusarium* lectin in presence Gdn-HSCN at pH 8.0[a]

Gdn-HSCN [M]	$-\Delta S_m$ ($J \cdot mol^{-1} \cdot K^{-1}$)	T_m (°K)	$-\Delta H_m$ ($kJ \cdot mol^{-1}$)	ΔG ($kJ \cdot mol^{-1}$)
0.05	335.2	330.2	108.6	4.17
0.1	297.3	327.3	97.3	3.35
0.2	285.7	326.4	93.2	3.07
0.3	252.1	323.7	81.6	2.33
0.4	240.1	321.5	77.4	2.10

[a] The value represent the mean of three independent sets of experiments with SD of less than 10%.

T_m was calculated from the plots of ΔG_U against T. ΔG (25 °C) was calculated using Equation no. (6).

$$\Delta G(T) = \Delta H_m(1-T/T_m) - \Delta C_p[(T_m-T) + T\ln(T/T_m)]$$

ΔC_p value used for calculation of ΔG (25 °C) was obtained from the slope of the plot of ΔH_m versus T_m varied as a function of guanidine thiocynate.

During thermal denaturation, many proteins do not refold, due to aggregation of the unfolded state (34,35). The aggregation usually involves unfolding of the whole protein or of a specific domain and it is often attributed to the association of partially unfolded molecules (36,37). Hence, aggregation stabilizes the unfolded oligomers by reducing the energetically unfavorable interactions between water molecules and exposed hydrophobic patches in the protein. However, in the present studies, static light scattering showed the absence of insoluble protein aggregates at higher temperatures and no ANS binding was observed.

The most important thermodynamic parameter is a large positive change in heat capacity of the two state F⇌U transition in protein denaturation, and within the range of experimental error, it may be assumed to

be a constant for a given protein (≈ 5 kJ·mol^{-1}·deg^{-1}) (38). This has been suggested to result from the exposure of hydrophobic patches in the process of unfolding due to contact with water (24). Changes in enthalpy of melting (ΔH_m) associated with thermal unfolding of the *Fusarium* lectin, varied linearly (r= 0.990) with melting temperature (T_m), resulting in constant value of heat capacity, ΔC_p, of the unfolding at different concentrations of Gdn-HSCN. This suggests that the heat capacity of the products (unfolded protein) was same as the heat capacity of the reactants (native protein) (39) and the unfolding did not result in change in the heat capacity of the lectin.

In conclusion, the above studies show that *Fusarium* lectin follows a two-state F⇌U unfolding mechanism during denaturation. The pH dependence of ΔG^{H_2O} suggested that the electrostatic interactions among the charged groups make a significant contribution to the conformational stability of the protein. At high pH, the lectin is more susceptible to chemical denaturants, while at low pH it shows flexibility in the tertiary structure with the retention of secondary structure and hemagglutinating activity.

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CHAPTER : 6

GENERAL DISCUSSION AND CONCLUSION

DISCUSSION

Several biological phenomenon such as fertilization, embryogenesis, cell migration, organ formation, immune defense and microbial infection are based on recognition and cell-cell interaction (1). Although lectins have been known for more than 100 years (2) only in the last two decades attention has been focused on the possibility that cellular recognition could be mediated by lectins. The study of role of lectins in fungal-plant interaction mainly revolved around the plant lectins and their role as defense tools of plants against various pathogens (3,4). Few studies have been directed towards understanding the role of pathogenic fungal lectins, in host-pathogen adhesion. The role of lectin in the endophytic fungus could be similar although disease symptoms are not manifested in the host.

Almost all the lectin containing phytopathogenic fungi studied are members of Deuteromycetes and few from Ascomycetes. These lectins from a similar group vary in their molecular weights, carbohydrate specificity and the physiological role played by them. In our studies, we have reported and characterized a lectin from *Fusarium* sp. (LR11). The species was isolated from stems of healthy rose plants. The genus *Fusarium* contains over 20 species most of them are pathogenic. The most common of these are *Fusarium solani*, *Fusarium oxysporum,* and *Fusarium chlamydosporum* (5). The *Fusarium* genera belongs to class Deuteromycetes of kingdom Fungi.

A lectin has been isolated from the of *Fusarium* sp. The maximum lectin activity was reported at 5^{th} day after inoculation. The fungus produced good amount of lectin when grown on MGYP medium (malt extract 0.3 % w/v, yeast extract 0.3 % w/v, peptone 0.5 % w/v and glucose 1 % w/v). The lectin was purified to homogeneity by conventional procedures involving hydrophobic followed by gel filtration chromatography. Most of the reported phytopathogenic fungal lectins like those from the *Rhizoctonia* genus (6-8) and Sclerotiniaceae family (9) have been purified by affinity procedures with the exception of *Sclerotium* rolfsii lectin (10) which has been purified by conventional chromatographic procedures similar to *Fusarium* lectin. We also made efforts to purify the *Fusarium* lectin by affinity chromatography on

Fetuin-Sepharose but the binding was too strong, and it could not be eluted under native conditions.

Fusarium lectin was found to be a homodimer of 26 kDa with subunit molecular mass of 13 KDa. The fungal lectins generally do not exhibit high molecular masses (15-90 kDa), could have more than one subunits which may or may not be identical. The subunits of the fungal lectins are usually held together by non-covalent interactions but the subunits of *Lactarius lignyotus* (11) and *Phallus impudicus* (12) are linked by disulphide bridges. The lectins from *Sclerotium trifoliorum, Sclerotium rolfsii,* (9)*, Boletus satanas,* (13) and *Laccaria amethystea* (14) are monomeric with the molecular mass of 32 kDa, 45 kDa, 63 kDa, and 16 kDa respectively. *Rhizoctonia crocorum* (7) lectin is tetramer with the molecular mass of 44 kDa. *Pholiota aurivella* is an polymeric lectin containing 6 or more subunits (15).

The *Fusarium* lectin is a glycoprotein with 3.9 % neutral sugar content. Lectin from *Beauveria bassiana* (16) contains 12.6% neutral sugar but the lectins from Sclerotiniaceae (9) and the *Rhizoctonia* (6-8) are not glycosylated.

Fusarium lectin does not agglutinate untreated and trypsinized human ABO type erythrocytes but agglutinates pronase or neuraminidase treated ABO erythrocytes. However, the *Rhizoctonia solani* (6), *Rhizoctonia crocorum* (7) and *Athelia rolfsii* (7) lectins agglutinated human, rabbit and pigeon erythrocytes. In contrast to other plant pathogenic fungal lectins, *Sclerotium rolfsii* lectins (10,17) agglutinated certain gram negative bacteria and yeasts but not human red blood cells.

The *Fusarium* lectin retains its hemagglutinating activity upto 70 °C. Most of the other pathogenic lectin are stable upto 50 °C but lectin from *Aspergillus niger* (18) shows high thermostability and is active upto 100 °C. Comparatively mushroom lectin are much more thermostable as *Ganoderma capense* (19) and *Polyporus adusta* (20) lectins are stable upto 100 °C and 70 °C respectively. The *Fusarium* lectin like other phytopathogenic fungal lectins retains maximum activity over a broad range of pH (6,7,16,17).

Fusarium lectin is a basic protein with a pI of 8.7. The fungal lectins show very high diversity in the pI, ranging from a minimum pH 4.3 for *Clitocybe nebularis* (21) to maximum upto pH 10.65 for *Auricularia polytricha* (22). Divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+} and EDTA did not affect the *Fusarium* lectin activity. Most of the fungal lectins do not require metal ions. The activity of *Beauveria bassiana* (16) lectin is also metal ion independent, whereas *Sclerotium rolfsii* lectin activity is inhibited by EDTA (1 mM) and required either Mn^{2+} or Ca^{2+} (1 mM) for its activity (17). *Polyporus adusta* lectin showed two fold increase in titre value in presence of 10 mm $FeCl_3$.

Amino acid composition of *Fusarium* lectin shows 37 % hydrophobic amino acids, 16 % glycine and a low content of sulphur containing amino acids. Similarly, the Sclerotiniaceae lectins also show abundance of asparagine/aspartic acid, glutamine/glutamic acid, threonine, serine and lysine and small amounts of sulphur containing amino acids and some lectins of Sclerotiniaceae do not have methionine or cysteine (9). The resemblance between Sclerotiniaceae lectins and the lectins from *Rhizoctonia solani* (6), *Rhizoctonia crocorum* and *Athelia rolfsii* (7) is in addition to the abundant acidic amino acids, presence of large amounts of lysine and only traces of methionine and cysteine. The N-terminal sequence of *Fusarium* lectin did not show similarity with any known lectins. Fungal lectins generally show high diversity in the N-terminal sequence, followed by some conserved sequences.

Hemagglutinating activity of *Fusarium* lectin was not inhibited by any of the simple sugars, disaccharides, oligosaccharides but only glycoproteins and some plant polysaccharides. The desialated forms of the glycoproteins are more efficient inhibitor than the sialated counterpart. Fungal lectins isolated from *Sclerotium rolfsii* (23) and *Beauveria bassiana* (16) interacts with glycoproteins. However, *Rhizoctonia solani* (6) lectin was inhibited only by simple sugars as N-acetylgalactosamine, galactose, mellibiose, raffinose and others, but not by glycoproteins as fetuin, asialofetuin, ovomucoid and thyroglobulin.

Active site characterization studies of *Fusarium* lectin have shown the

involvement of tyrosine residue in the sugar binding site. Tyrosine has been implicated in the sugar-binding activity of lectins from fungi *Ischnoderma resinosum* (24) and *Hericium erinaceum* (25); and plant lectin as from *Artocarpus hirsuta* (26), *Erythrina indica* (27) and *Trichosanthes dioica* (28).

The sugar binding properties of the *Fusarium* lectin was explored in details using spectrofluorimetry and surface plasmon resonance. Agreement between the data obtained by this two technique substantiate the monophasic nature of binding. *Fusarium* lectin was found to interact Gal/GalNAc residues present in disaccharides and glycopeptides. Wu *et al.* have (29) have grouped Gal/GalNAc specific lectins according to their specificity for the disaccharides (as they are part of bigger structures):

Class F- Forssman specific disaccharide (GalNAcα1→GalNAc)

Class A- Human blood group A specific disaccharide (GalNAcα1→3Gal)

Class Tn- GalNAcα1→*O*Ser/Thr

Class I/II- Human blood group type I/II precursor sequences (Galβ1→3/4GlcNAc)

Class T- Galβ1→3GalNAc

Class B- Blood group B specific disaccharide (Galα1→3Gal)

According to this classification the *Fusarium* lectin belongs to class T as well as Class I/II, and the order of reactivity is Galβ1→3GalNAc > Galβ1→4GlcNAc.

In the SPR studies, *Fusarium* lectin showed several fold higher affinity for the glycoproteins ($K_a \approx 10^6$-10^{10} M^{-1}) than glycans ($K_a \approx 10^3$-10^4 M^{-1}). The lectin from mushroom *Psathyrella velutina* showed K_a in the range of 10^7-10^8 M^{-1} with fetuin and asialo-agalactofetuin (30). The interaction between *Psathyrella velutina* lectin and fetuin or transferrin implicated the involvement of terminal sialic acid residues in the lectin binding, although it also exhibits higher affinity for asialo-agalactofetuin than fetuin (30).

The kinetics of binding of glycans and glycoproteins to *Fusarium* lectin followed by SPR reveals that the binding process follows a bimolecular

one step mechanism. The association rate constants are much slower than that for diffusion controlled reaction. High affinity of lectins for glycoproteins is accompanied by their faster association rates ($k_1 \sim 10^5$ $M^{-1} \cdot s^{-1}$) and slower dissociation rates ($k_{-1} \sim 10^{-3}$ s^{-1}). *Psathyrella velutina* (30) and *Fusarium* both the lectin showed similar rates of association and dissociation.

Effect of different chemical and physical denaturants such as urea, guanidine thiocynate, temperature and pH on structural stability of *Fusarium* lectin was monitored by fluorescence spectrometry and circular dichroism. The lectin showed considerable structural stability for urea, whereas guanidine was very effective denaturant. The free energy required for lectin unfolding was comparatively low than other lectins. Frutalin (*Artocarpus incise* lectin) a tetrameric lectin showed conformational stability of 17.12 $kJ \cdot mol^{-1}$ and 12.34 $kJ \cdot mol^{-1}$, in the presence and absence of D-galactose, respectively (31). Unfolding process of *Fusarium* lectin was irreversible, whereas the unfolding process of galectin-1 (from porcine spleen) was reversible (32). Surolia *et al.* (33) and Schwarz *et al.* (34) investigated thermal unfolding transition of galectin-1, describing tetrameric and octameric folding intermediates. However, such intermediates were not observed in the unfolding studies of *Fusarium* lectin. KM+, a lectin isolated from *Artocarpus integrifolia* showed very high structural stability against urea, guanidine hydrochloride and SDS at physiological temperatures. Although temperature was found to be an effective denaturant, since the lectin starts to loose its conformation above 60 °C (35). Monomeric intermediates, termed as molten globule, retaining carbohydrate recognition property have been reported for lectins. A molten globule stage was observed during guanidine hydrochloride induced denaturation in peanut lectin, which retains its carbohydrate binding property (36). Such active intermediate state was also observed for concanavalin A, and it appeared in 30 % (v/v) polyethylene glycol (37). The *Fusarium* lectin, however, did not show formation of any intermediate or molten globule.

Lectins are a family of proteins of unknown function and the challenge of identifying the physiological function for these proteins still remains. Biochemical approaches have resulted in extensive data on the structure and molecular properties of lectins. The elucidation of lectin function will require synthesis and correlation of data obtained from the study of many lectins. In this way, information regarding specificity of a fungal lectin from *Fusarium* with different glycans and glycoproteins would be helpful in understanding the function of the lectins in the pathogenecity of the fungus.

CONCLUSIONS

1. A lectin was isolated from the mycelia of *Fusarium* sp. (LR11/NCIM 1330).
2. The lectin is a homodimeric, glycoprotein with a molecular mass 26 kDa and pI 8.7.
3. The lectin is thermolabile, since it lost its hemagglutinin activity up to 30 °C but showed considerable pH stability over a broad pH range 2-12.
4. Active site characterization studies show the involvement of tyrosine in sugar binding.
5. The lectin exhibits specificity for complex glycan and glycoproteins containing Galβ1→3GalNAc or Galβ1→4GlcNAc. It also shows inhibition with plant gums such as gum ghatti and gum karaya.
6. It exhibits moderate conformation stability, guanidine thiocynate acts as an efficient denaturant for lectin, whereas temperature, urea and pH have only marginal effect.

List of publications :

1. Purification and characterization of a lectin from endophytic fungus *Fusarium* sp. (NCIM 1330) having complex sugar specificity
   Feroz Khan, Absar Ahmad and M. Islam Khan (Communicated).
2. Interaction of *Fusarium* sp. (NCIM 1330) lectin with complex glycans and glycoproteins: A surface plasmon resonance study on role of protein matrix in lectin-glycoconjugate interaction.
   Feroz Khan, Arvind Sahu, Absar Ahmad and M. Islam Khan (Manuscript under preparation).
3. Equilibrium unfolding of a lectin from *Fusarium* sp. (NCIM 1330) : Effect of urea, guanidine thiocynate, pH and temperature.
   Feroz Khan, Absar Ahmad and M. Islam Khan (Manuscript under preparation).

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